

Transparency at Stockholm

The conference opening this week at Stockholm is another opportunity for arms control.

It should keep hopeful ambitions in check and concentrate, instead, on mechanisms for monitoring.

ON the principle that nuclear war is too important to be left to the nuclear superpowers, European states must make the most of the Conference on Disarmament in Europe which opened this week in Stockholm. The signs are that the countries concerned recognize the occasion as an opportunity. But so, it seems, do the Soviet Union and the United States, both of which seem to have taken fright at the breakdown of the arms control negotiations at Geneva at the end of last year, and which have been making noises that may be construed as conciliatory in the past few days. (More accurately, the United States has been making contradictory noises, not unalloyed denunciation, while the Soviet Union has lapsed into silence.) Rarely can states which have by muddled mismanagement lost one opportunity (see *Nature* 306, 111; 1983) so quickly been provided with another.

The sunny climate in which people appear to have assembled in Stockholm does not, however, guarantee a successful outcome. Indeed, the constitution of the conference is hardly that of a gathering at which tangible agreements could be reached on matters that have not already been fully explored. Altogether, 35 countries will be represented at Stockholm, all of them signatories of the Helsinki Agreements signed in 1975. The gathering is in this sense a kind of relic of a quite different epoch, that in which East-West detente was the touchstone for most international relationships. Almost as they were signed, the Helsinki agreements came to seem more trouble than they were worth. Various people in the Soviet Union, Brailovsky for example, found themselves locked up because they formed the Helsinki Monitoring Group to ensure that high-sounding language agreed at Helsinki was being translated into reality. (Broadly speaking, people's freedom to move from place to place has not been upheld.) As detente withered, a process that began long before the Soviet hegemony over Afghanistan, the successive conferences held within the framework of the Helsinki agreements became increasingly acrimonious, more like opportunities for threatening the withdrawal of cooperation than the recurrent use of that word in the Helsinki documents implied. The meeting that ended last year in Madrid, and which led directly to this week's encounter in Stockholm, showed how ungainly a forum for negotiation such occasions can be. Various, it was an occasion for mutual recrimination, for flying adventurous but improbable kites — and for some smaller members, Malta for example, to advertise their presence by demanding satisfaction on some peripheral issue as the price of agreement on the date of the next meeting.

Agreement

Helsinki has, however, produced one tangible understanding which is potentially of great benefit to European security, East and West. This is the agreement between all parties that military manoeuvres within 150 kilometres of the boundary between East and West will be notified in advance, and that observers will from time to time be invited by the government concerned. There have been several occasions in the past five years when this facility has helped to steady people's nerves. The fixed element of the agenda for Stockholm is a plan, derived from what was originally a French proposal, to extend this arrangement to cover the whole land surface of Europe and, at the same time, to ensure that military manoeuvres are open to observers from the other side as of right, and not at the discretion of the countries concerned.

In the absence, perhaps a long one, of an agreement on nuclear weapons based in or aimed at Europe (East and West), the prospect of such a lesser agreement is bound to be attractive. Anything, people will say, is better than nothing — and they will be right. Moreover, if the principle can be agreed that all European governments should know what others are doing with their visible military forces, the way may be opened for a more general understanding that the same principle should apply to the more remote strategic components in the uneasy balance — strategic missiles, long-range aircraft and even lesser weapons of special kinds, nuclear artillery shells for example. That seems to be the objective in the minds of the governments now advocating the benefits of what they call transparency as a first substantial step towards mutual security. The question that must be tackled seriously in the weeks ahead is whether the mechanisms provided by the Helsinki agreements are durable enough.

Conviction

As with all agreements between mutually distrustful governments, it is dangerous to let good intentions outstrip the means of telling that they have been realized. So much is clear from the professed conviction of the United States Administration that the Soviet Union has violated existing agreements on arms control. The administration has in the past few days been telling people in Congress of these transgressions in ways calculated to ensure that what it has to say finds its way into the newspapers before it formally makes them public next week. The snag, with all these allegations, is that the data published to support them are unconvincing. The claims that South-East Asian yellow rain is a vehicle for delivering mycotoxins to innocent people are, as published, unconvincing — but the US State Department falls back on unpublishable intelligence information said to prove the case to the hilt. Much the same is true of the allegations that the Threshold test-ban treaty of 1974 has been violated by the Soviet Union; the data so far published are not necessarily inconsistent with the threshold limit of 150,000 tonnes of TNT equivalent that specified the threshold — but, ironically, the uncertainties might well be removed if only the two sides would formally ratify the treaty and then, as required, exchange the data about seismic travel times in their nuclear testing grounds on which a more accurate assessment might be based. The moral is that the plans now being hatched at Stockholm should be as much concerned with means of verifying whatever may be agreed as with the objectives of agreement.

This, unfortunately, is where the trouble starts. Even the agreement that military manoeuvres in Europe should be notified has been challenged, perhaps inevitably. The objective of any agreement along these lines is to ensure that one government will not mistake another's decision to exercise its military forces as a sign that some kind of attack is imminent, perhaps prompting pre-emptive measures. But who can tell, simply by looking at what happens, the difference between manoeuvres which must be notified and substantial movements of troops and military equipment in the normal course of business, perhaps because of a strategic decision that forces should be differently disposed? This is why the transparency that has become the objective of many European governments will be persuasive (and safe) only if there is some mechanism for making sure that a government suspecting



the worst will have access to means of verifying the facts of what may seem a threatening development and then of coming to an understanding of why that development has taken place.

The obvious mechanism is to arrange that a complainant government should have the right to question any other about its intentions, ideally as part of some framework of regular meetings. The trouble is that there will be natural limits on the frequency with which awkward questions may be raised arising from political considerations and even from the unwillingness of governments implicitly to disclose the weaknesses of their intelligence networks. So why not replicate for the monitoring of European forces a system comparable with the nuclear safeguards inspectorate, which in the past decade has made the Nuclear Non-Proliferation Treaty work without substantial complaint? The result, it is true, would be yet another international body staffed by international civil servants, but the value of such an organization would far outweigh not merely its cost but the irritation it would no doubt cause. Moreover, because such a body of inspectors, individually nationals of member states but collectively impartial, would serve as a model for the supervisory authority there would have to be in any more ambitious programme for transparency in Europe than that now planned, its creation would be a more important gain than any other agreement that could be quickly reached at Stockholm.

Mercifully, such an arrangement would also fit in well with the growing belief, at least in Western Europe, that safety from the threat of all-out war cannot much longer be left to the two principal nuclear powers. Little benefit would result from a broadening of the basis of the negotiations on nuclear arms which have broken down at Geneva, which is not to say that the British and French nuclear forces are irrelevant. For if two powers fail to reach agreement, the chances that four (or even five, with China) would do so must be smaller. Yet the construction of some international mechanism for supervising the movement of soldiers and their equipment (and in due course of counting stockpiles?) would go a long way to satisfy European governments that they have a voice in matters which affect them vitally, but over which they have no direct control. □

Pork-barrel supplement

The US Administration hopes to spend more on competitive grants in agriculture. It must insist.

THE word has gone out from those within the Reagan Administration who support competitive grants for agriculture that good news is coming at the end of the month, when the President will release his budget proposals for fiscal year 1985 (see p.204). The competitive grants programme in the US Department of Agriculture (USDA) has been held to a ludicrously low level — \$17 million — by political interests which see perfection in the century-old share-the-wealth system that passes for an agricultural research structure in the United States. Every state gets a piece of the action, no questions asked. Congressmen pat themselves on the back for bringing home the goodies. And mediocre researchers continue to grind away at the same old applied technology that might have made sense once upon a time.

The message has finally begun to sink in, however, that questions must be asked if agricultural research is to remain in touch with twentieth-century science. USDA is said to have asked for \$50 million for competitive grants for 1985. The obstacle now appears to be the Office of Management and Budget, which is thought to have ensured that only half of that request will see its way to the budget. The administration last year proposed \$21 million, which Congress cut back to \$17 million — the same level as for years past. The route to real change is not easy. The administration will have to battle with entrenched interests in Congress, particularly one Representative Jamie Whitten of Mississippi, chairman of the House Appropriations Committee and a stalwart defender of mediocrity when it is in the interest of his home state. A half-hearted effort by the administration will not do. It will have to have the courage of its newly-found convictions. □

Examination fever

British school education is in for another shake-up. The minister in charge should move carefully.

SIR Keith Joseph, Secretary of State for Education and Science, has been in his job for what by British standards is a suspiciously long time. Even if the explanation is that there is nowhere else in the government for him to go, the consequence is beneficial. Familiarity with the educational system he administers has now led Sir Keith into a radical reappraisal of how the job should be done. At a conference in Sheffield earlier this month, he announced that his department plans to establish objectives for the main parts of the school curriculum in British schools for children aged from five to sixteen. By doing so, he has issued the most important challenge so far to the doctrine underlying the 1944 Education Act — that central government pays for the cost of public schooling, that local education authorities administer schools and influence the curriculum as they can — but that the final arbiters of what is taught are teachers.

This doctrine has increasingly become a fiction in the past four decades. Teachers have done the best they could in a quickly changing world, but have been given too little opportunity to keep up with what they hope to teach. Local education authorities have sometimes done what they could to help, but their performance has been patchy. Bodies such as the Schools Council, conceived of as an instrument for curriculum development but now demolished, have spent too much of their energy on procedure and not enough on content. Central government has had to be content with seeking to influence the system by the reports of advisory committees appointed for special purposes or by the much diminished influence of its inspectors. So, by default, the school curriculum has in effect been determined by the uniquely British examination system. That, it seems, is the discovery which Sir Keith has now made and the circumstance that he seeks to change. The result could be invaluable, especially in the teaching of science.

As things are, much of the British public is not merely ignorant of even the most basic science but actively suspicious of it. Science is often regarded as a necessary evil that will corrupt the minds of children as they lose the innocence of youth, but that children of primary school age should be exposed to such adult material, heaven forbid, seems quite shocking. Even at secondary level, the notion persists that science is an eccentric pastime or, even more demeaning, a vocational pursuit — a device to enable clever children to pass difficult examinations but remote from the concerns of everyday life. Such effete-ness has persisted because, astonishingly, it has never been thought necessary in Britain to specify the basic concepts and information about the world that the school system should aim to teach. Science, while widely taught, is not an essential part of every student's education. Now that may change.

Sir Keith Joseph's intention seems to be to determine what is taught in British schools by reforming the examinations system. There can be no sensible objection to such an attempt, except perhaps the government's desire at the same time to find short-term economies in public expenditure. The argument that teachers should be allowed to adapt their teaching to local needs is no objection, being an obvious truth and not an alternative. That an education system paid for by the British taxpayer should conform with nationally agreed objectives is self-evident.

Yet as things stand, the system does nothing of the sort. At secondary school level, the curriculum is hagridden by examinations. As things are, there are two tiers of school-leaving examinations, one for those seeking places at universities called A ("advanced") level and taken at age eighteen or thereabouts, and a second layer of two examinations at sixteen, called O (for "ordinary") level and CSE (for "Certificate of Secondary Education") respectively. Three years ago, the government agreed to follow its predecessor's undertaking that the examinations at sixteen would be combined, but the details are still being worked out.

Meanwhile, the boards that set examinations for sixteen-year-olds continue to devise examination questions that produce a good spread of results correlating positively with the results of other examination questions and which also satisfy the (counter-productive) demand from employers for a wide spread of grades by cramming in such a range of questions that teachers are hard-pressed to cover the syllabus in time. Not surprisingly, education in general, and science education in particular, suffers: the examinations test stamina and quick-wittedness as much as any skill applicable in real life. At primary school level, science education depends on one thing only: whether there happens to be a teacher in the school who knows anything at all about science. Often there is not.

Not content with challenging the historical basis of the school curriculum, Sir Keith Joseph wants also to introduce public examinations that record absolute levels of attainment ("criterion referencing") to replace the present system, which is based largely on the assessment of performance relative to that of others ("norm referencing"). Practical difficulties aside (who could decide an absolute criterion for, say, appreciation of English literature?), this proposal could easily become a red herring. The year on year correlations made by examining boards ensure that the present system is not totally lacking in objectivity. But the real reason why the present two-tier system of examinations at 16-plus urgently needs unifying is that it is unjust.

The CSE examinations intended for the middle 40 per cent of the ability range have, over the years, become more and more similar in content to those (O levels) intended for the top 20 per cent of the ability range. There are no examinations designed for the 40 per cent of students at the bottom of the ability range although, under pressure from parents and teachers, many of them do attempt CSE. For similar reasons, many who should be taking CSE take O levels. The interest of the student is not best served by pressing him to take an examination in which he will probably score badly. To add insult to injury, those who do obtain high grades in CSE are often deemed by ignorant employers to be inferior even to failed O level candidates. In Sheffield, Sir Keith Joseph seemed to be hinting that he might back away from a commitment to establish a unified system of 16-plus examinations. The clear need is for a single set of achievement-related tests that would be of real value to future tutors and employers alike. An attempt to compromise on this issue could easily be disastrous.

What is it all for? Sir Keith Joseph says he intends to improve educational standards to the point where 80–90 per cent of students achieve the levels now reached by pupils of average ability at age sixteen. The variation of examination performance between schools suggests that this ambitious goal is feasible. To succeed, however, the goodwill of the teaching profession will be needed. Sir Keith's speech in Sheffield, in which he went out of his way to compliment teachers in general, is a welcome sign that he recognizes that reality.

But teachers will need more than a pat on the back. Science achievement in schools is being constrained by inadequate resources, as shown by studies carried out by the Department of Education and Science's assessment of performance unit, while the annual reports of Her Majesty's Inspectors of Schools show that, for example, 60 per cent of schools are inadequately provided with textbooks and 20 per cent have serious equipment shortages. It is arguable that some local education authorities are not fulfilling their statutory obligations under the 1944 Education Act. Even more important, shortages of teachers in key subjects (especially mathematics and physics) have meant that many vacancies are being filled by inadequately qualified people. Poorly motivated and under-confident teachers are unlikely to be an inspiration to their charges.

The reason for this state of affairs is not hard to find. Two years ago, a committee under a the chairmanship of Dr Wilfred Cockcroft established that qualified mathematics teachers, for example, then earned £1,000–£2,000 less per year than their counterparts in industry and commerce. Since that time, circumstances have deteriorated, and the present salary structure of

teachers is made incoherent by the problem of falling school rolls.

It is perhaps a blessing that the long recession is not ending quickly, for then the teachers now in posts requiring special skills would be snapped up by industrialists out head-hunting. Sir Keith Joseph now seems to recognize that this problem will not disappear unless there are more resources in the system to keep teachers at their desks — and seems willing to press for extra. Not before time, it will be agreed.

Part of the trouble with the British school education system is that it is commonly regarded as a system on its own, with nothing in common with educational systems elsewhere. Yet this is precisely the time when, in the United States, the federal government has also woken up to the crisis in the schools, has uttered a series of clarion calls for improvement — and has found that the managers of the school systems are ready to respond (at least for a time). In both countries, the underlying problems are very similar, as they are in industrialized Western Europe as well: increasing professionalism in adult work, and the increased demands of the school curriculum, make teaching seem a backwater profession. If it is underpaid as well, the consequences can be calamitous. Teachers with professional skills outside the classroom will simply melt away — or worse, will be replaced by teachers who claim but in reality lack them. In the United States, many school systems have seen the way the wind is blowing, and have chosen to pay the teachers they need to keep above the odds. In Britain, the system of nationally negotiated salary scales will make the solution more expensive. But there is no choice.

That is one battle the minister will have to fight. Another, closer at home, is the battle he will have to fight within a government which, throughout the past five years, has been even-handed in its meanness towards the different sectors of the educational system but, at the school level, has taken advantage of falling school rolls hugely to economize. Now, as the minister seems to have realized, the process has gone so far that students are being denied opportunities that they, and ultimately the rest of us, would profit from. But at a time when public economy is still all the rage (and economically necessary as well), will he be able to wring the funds he will need from his colleagues, and if so, at whose expense? □

You are not what you eat

Too much cholesterol in blood is bad for people; the same is not true in food.

A NEW study from the National Institutes of Health (NIH) on the benefits of lowering cholesterol levels has predictably set off another wave of bad advice from the dietary pundits who have never been able to distinguish between cholesterol in food and cholesterol in blood. What the study did show, and show conclusively, is that high-risk patients — those with blood cholesterol levels over 265 mg per dl — are significantly less likely to have a heart attack if they can reduce those levels to something closer to the US national average of 210 mg per dl. Almost 4,000 men, all members of this high-risk group, were tracked for more than 7 years; all followed a reduced-cholesterol diet, but half were in addition given a drug to reduce blood cholesterol. The test group, which reduced its blood cholesterol levels by about 17 per cent on average, had 24 per cent fewer fatal heart attacks and 17 per cent fewer non-fatal heart attacks than the control group, which managed only a 3.5 per cent reduction in blood cholesterol.

What the study did not show is that the general population would benefit at all by restricting cholesterol in the diet. Early studies, indeed, have shown that it makes little difference whether one's blood cholesterol level is 210 or 230 mg per dl — the benefits that come from reducing blood cholesterol are far from linear. Dietary cholesterol, according to this and earlier studies, makes too little difference to those who really need to do something about the cholesterol in their blood (and who, the NIH study suggests, may well benefit from drug therapy) and is simply irrelevant to those with average cholesterol levels. □

Fast breeder reactors

Europeans sign joint development programme

THE United States may have abandoned Clinch River, but Europe last week confirmed its commitment to the fast breeder reactor. At least, that is one interpretation of an agreement signed on Wednesday by five European states — the United Kingdom, France, West Germany, Italy and Belgium — to open the way to cooperation on fast breeder research and development and commercialization. A more sanguine view is that the agreement is largely a way to reduce the embarrassing £300-million a year cost of European work on fast breeders.

Britain, for example has already announced a cut in its fast breeder programme. Britain's present spending of £108 million a year (1983–84) will fall by a third by 1986–87, according to a spokesman for the UK Atomic Energy Authority, notwithstanding the new agreement (which does not mention cash figures).

Moreover, Peter Walker, Britain's Secretary of State for Energy, said in announcing the agreement that the fast breeder potential (for energy supplies next century) "can be developed only if Europe . . . is assured that it would provide an economic supply of energy". The memorandum is directed towards this end, he said, and to assuring safety. At the moment, fast breeder electricity is not economic, the costs being estimated to be about double those of a thermal pressurized water reactor.

Nevertheless, the other major partners are pushing ahead rapidly with their own fast breeder construction programmes. In the south of France near Avignon, the 1,200 MWe Superphénix reactor, cooled by a great basin of liquid sodium, nears

connection to the grid, and at Kalkar in West Germany the SNR 300, cooled by a sodium loop, should be completed in 1985, and connected to the grid in 1987. The German Federal Government claims to have solved the problem of financing Kalkar by transferring the bulk of construction costs to industry — but it is still financing the fast breeder research and development team at Karlsruhe. This team should also be supported by industry, the government argues, and it hopes that the companies will agree.

Meanwhile the international agreement signed last week will enable the transfer of know-how, technologies and people among the partners. "It would be absurd", said Mr Walker, "for various countries in the European Community to duplicate the research and development work that is needed. The work can be done more effectively and more economically by working together."

Exactly what economies will follow is not clear, but the agreement signed last week is to be followed at an early date by other more detailed agreements covering research and development, industrial cooperation, patenting and industrial property, fuel cycle arrangements (including transport and processing of plutonium) and an inter-utility agreement "to provide for the joint realization of fast reactor power stations and the exchange of operational experience".

It is not thought likely that Britain, at least, would consider building a successor to Superphénix for upwards of 10–15 years. France, too, may find it difficult to support, with the national utility EDF currently facing international debts of FF150,000 million (£12,500 million) and a surplus of electricity.

Robert Walgate

French fast breeder reactors

Military doubts in France

COULD the new European agreement on fast breeders help France to make more nuclear weapons? On the face of it, such a military interpretation would fly in the face of the agreement, a "memorandum of understanding" which announced that signatories "confirm that activities taking place as a part of this collaboration will be directed to the peaceful development of nuclear energy".

Nevertheless, simply to maintain the security and future of the French fast breeder programme — the main component of European work — is also to maintain the French weapons programme, a vociferous group of French scientists is claiming.

Jean-Pierre Pharabod of the Ecole Polytechnique, for example, claims that the 40 MWth Phénix reactor — a prototype fast breeder power station now to be succeeded by Superphénix — has been used for military plutonium production. Pharabod also says that this fact has been communicated by France to the director of Euratom, the European treaty organization for nuclear research, based in Brussels.

Certainly, France has not submitted inspection of Phénix to the International Atomic Energy Agency (IAEA) — unlike the UK Atomic Energy Authority's Dounreay fast breeder, the PFR, to which IAEA inspectors have been admitted. According to Pharabod, the French military actually need Superphénix (a 1,200 MWe station due on line next year) to fulfil their planned (and announced) expansion of the French deterrent. The number of submarines, now five, each equipped with 16 missiles each with a single warhead, is to increase to seven submarines with the same number of missiles but each with six warheads; and there will be some 200–400 new tactical weapons with yields of some 100–300 tonnes of TNT. (For example, 120 Hades missiles will replace the present 40 Pluton ground-to-ground weapons.)

These developments will need new plutonium, and the arithmetic shows that only Superphénix can provide it, says Pharabod. Thus it is impossible in France to disentangle peaceful from military uses of plutonium, however much the new memorandum of understanding might appear to do so.

Indeed, according to a statement to the French Parliament by a socialist deputy, M. Georges Benedetti, who represents the nuclear area of Marcoule, "the fast breeder is the best choice for assuring both national energy independence and military independence . . . The fast breeder is the only means now of making plutonium of more than 95 per cent Pu-239 in sufficient quantities to feed the development of our nuclear forces".

Robert Walgate

Asian academy established

New Delhi

A FEDERATION of Asian Scientific Academies and Societies has formally been established with the long-term aim of "solving regional problems without disturbing the natural ecosystem". The idea of such a federation was formulated at a meeting sponsored by the Indian National Science Academy (INSA) two months ago. Appropriately, the formalities setting up the 10-member federation were completed on 15 January, the day before the golden jubilee celebrations of INSA.

INSA, the oldest of India's science academies, though not of its learned institutions (the Calcutta-based Asiatic Society has just celebrated its bicentenary), is the organization responsible for India's

international scientific cooperation programme. Guests at the celebration therefore included representatives from many national academies as well as the American Association for the Advancement of Science and the Royal Society of London, all of whom were to address the two-day symposium following the jubilee session.

The main issue at present facing Indian science — the balancing of the pursuit of excellence against the immediate needs of the country — was, however, the keynote of the jubilee session. Professor A. K. Sharma, president of INSA, stressed the need to ensure the academy's autonomy by guaranteeing it an annual budget that would be stable in real terms.

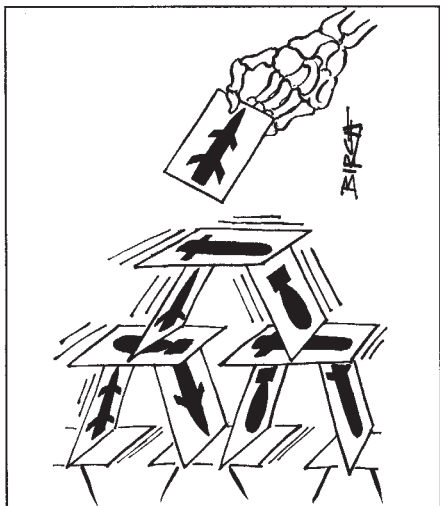
Vera Rich

US nuclear arsenal

Eight new warheads a day

Washington

MORE than 700 US combat units — over 100,000 troops — are “certified” in the use of nuclear weapons, according to a compendium of US nuclear forces published last week by the Natural Resources Defense Council (NRDC)*. The book, a sort of illustrated catalogue of the US arsenal, offers an unusually complete picture of the full array of tactical and strategic weapons maintained by the



United States and of how deeply integrated they are into the US fighting forces. All four military branches — plus the Reserves and National Guard — are trained to use nuclear weapons. Approximately one-half of the 26,000 warheads in the US stockpile are designed for use in battle-field weapons, including antisubmarine and

anti-aircraft rockets, air-to-air missiles, artillery shells and even land mines.

Neither the Department of Defense nor the Department of Energy (which has responsibility for production of nuclear warheads) would comment on the accuracy of the data in the book, which was compiled from open sources, including congressional testimony and data obtained under Freedom of Information Act requests. The Department of Energy, however, did advise the authors that publication of the book would be damaging to “the national interest”, although it stopped short of taking any legal action to halt publication or distribution.

Some of the most interesting data in the book deal with tactical weapons, which, as co-author Milton Hoenig notes, are not covered even under the stalled SALT and Intermediate Nuclear Forces negotiations. The United States has deployed 5,000 nuclear artillery shells, mostly in Europe, as well as surface-to-surface guided missiles, anti-aircraft missiles, and two varieties of nuclear land mines, known as “Atomic Demolition Munitions” (ADMs). The smaller of these ADMs weighs about 60 pounds and is intended for use by commandos against targets behind enemy lines. It has an explosive yield of as little as 10 tonnes (TNT equivalent). A test of an ADM at the Nevada Test Site in 1955 nonetheless produced a crater 400 feet across and 60 feet deep. Three artillery warheads, for 155-mm and 203-mm guns, are in use, including “enhanced radiation” devices.

The Navy maintains the greatest variety

of short-range nuclear arms: ship-launched and submarine-launched antisubmarine rockets, anti-aircraft missiles and surface-to-surface missiles. The Navy is also developing a new air-to-air missile, the Phoenix, to be carried by carrier-based long-range interceptor planes.

The book also provides data on nuclear weapons production, which is now proceeding at the pace of eight new warheads per day; taking into account the warheads retired from service, the result is still a net gain of three per day. According to the book's authors, the US stockpile will grow by 13 per cent over the next five years; by the mid-1990s the arsenal will be approaching the previous peak of 32,000 warheads (reached in 1967) and will in addition have been thoroughly modernized, most warheads having been replaced by new, higher-yield versions.

Future volumes from NRDC will deal with the Soviet nuclear arsenal, arms control and nuclear strategy.

Stephen Budiansky

**Nuclear Weapons Data Book* by T. B. Cochran, W.M. Arkin and M.M. Hoenig (Ballinger, Cambridge, Massachusetts, 1984; \$19.95).

Nuclear reprocessing

West Germany plans ahead

THE West German Federal Ministry of the Interior last week reaffirmed its support for the construction of a reprocessing plant for nuclear waste, in particular “to secure national independence in this area”.

The organization responsible is the *Deutsche Gesellschaft für Wiederaufbereitung von Kernbrennstoffe* (DWK) which was formed jointly by the 12 electricity companies concerned. The proposed undertaking will be financed by DWK and will receive no money from the federal ministry.

Approval procedures are now under way for two sites: Dragahn in Nieder-Sachsen, not far from the Gorleben underground storage area, and Wackersdorf, Schwandorf in Bavaria. The procedures should end late this year, when DWK will decide which site it wants. Only final approval by the *Länder* ministries will then be necessary before building starts.

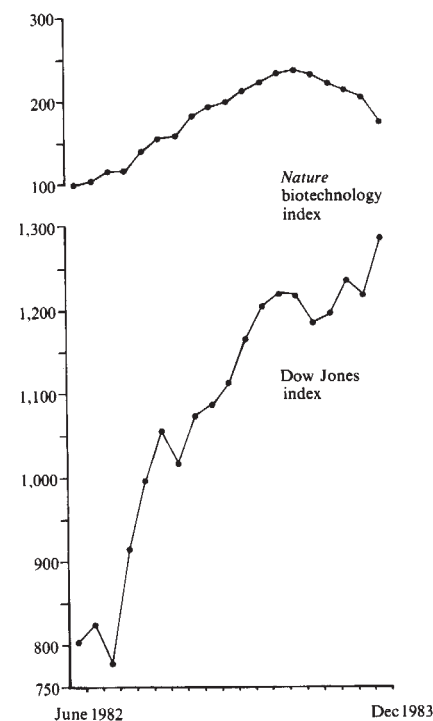
The plan is to build a single installation capable of processing an average of 350

tonnes of spent rods per year, beginning in 1986 and coming into service slowly from 1993 at a rate of 50 tonnes and working up to a final 450 tonnes annually. More than 500 tonnes would overstep environmental control limits.

Meanwhile, the question of costs is causing concern. Electricity from nuclear installations, although it is now cheaper than that from coal-fired stations, already carries a small surcharge to the eventual cost of reprocessing. The revenues collected will accumulate in a fund for the construction of the reprocessing plant. While the cost of the plant is currently given as DM4,300 million (£1,100 million), it could easily rise to DM6,000 million or DM8,000 million before completion, with annual running costs of DM1,000 million. “A reprocessing plant is not an economic proposition”, the industry's union spokesman asserted, but nevertheless supported the project as a demonstration of industrial expertise.

Sarah Tooze

The falling index



NATURE's monthly biotechnology index tumbled another 21 points in December 1983 (see 12 January, p.102), falling to 175 against the peak of 238 in July 1983. The index started at 100 in June 1982. During the same period, the overall value of shares on the US stock markets, as represented by the Dow Jones index, has risen dramatically (see graph). Moreover, the decline has been spread across the biotechnology board, affecting 15 of the 16 shares from which the index is compiled. □

Franco-Chinese collaboration

Tibetan expeditions draw breath

THE spectacular French–Chinese geological expedition to Tibet (see *Nature* 5 January pp.17–36), is unlikely to be repeated — at least for a few years. The problem is not one of politics but of resources according to the expedition's French organizer, Claude Allègre of the Institut de Physique du Globe in Paris.

The expeditions, which have revealed that the high plateau of Central Asia was created by a succession of intercontinental collisions and not just one, "have been a huge effort for both sides" says Allègre. There were three separate expeditions, in 1980, 1981 and 1982. Each involved 50 big trucks in the field, and 150–200 people. France put in some of its best geologists. Chinese resources — both people and cash — were especially stretched.

As a result, no further major French–Chinese expeditions to the area are

rectified much more cheaply. Moreover there were other plateaus in the world which were more accessible to western geologists, says McKenzie: for example eastern Turkey, the Colorado plateau, and the Altiplano. Also, relations with China were improving to the extent that person-to-person partnerships could now be arranged, McKenzie feels, rather than vast government-backed expeditions — and in fact McKenzie is going to China on such a partnership this Easter.

Allègre says he had sought European collaboration when there were plans to continue beyond the first three years, probably with other European countries taking up the running from France. But while individual geologists had shown great interest, none was prepared to organize the collaboration with the Chinese, Allègre claims.

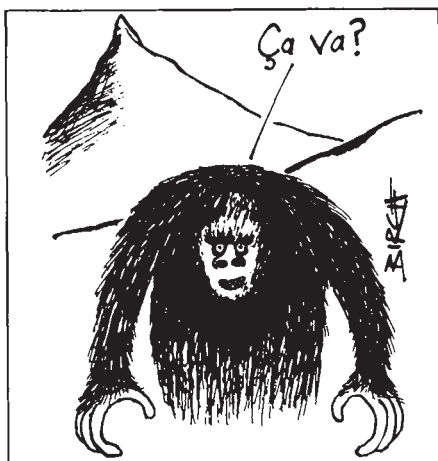
Perhaps this was wise. Allègre describes the negotiations that led up to the expeditions as "terrible", and as having been several times on the point of breaking

down. The Chinese drove a very hard bargain, which has led — for example — to the French team leaving several major instruments behind.

Allègre clearly does not relish entering such negotiations again. The result, however, was fair to both sides, he feels. Allègre says he cannot estimate the overall cost, but it had been shared equally, he claimed.

President François Mitterrand of France himself has shown real interest in the expedition, Allègre says, and by no means only in its politics. The President reveals a "greater and greater interest" in science, not only as a means to develop the French economy. Allègre, who accompanied Mitterrand on his 1983 tour of China, says that Mitterrand asked him more about plate tectonics than about science politics.

As a result, Allègre considers that while French science is not as well blessed as promised by the former research minister Jean-Pierre Chevènement, the budget is "protected", and will be in the future. Mitterrand has a very balanced view of the relation between basic and applied research, Allègre believes — more balanced than his ex-minister. **Robert Walgate**



planned, but Allègre believes that the Chinese will not be seeking collaboration with other partners either. Meanwhile, however — while both sides gather breath — France and China will mount small joint expeditions each year, the next to the Ping Ling mountains.

In any case, Allègre says, "we've understood what we wanted" about the region. While there is much more to learn, only a third of the results of the expeditions have been published so far and the teams need a few years just to think about the results.

Why had only France been involved in the expeditions, and not other Western countries? Partly, it may be because other geologists were less enthusiastic than Allègre about the exercise. Dan McKenzie of the University of Cambridge, for example, one of the pioneers of plate tectonics, says that while the expedition had been important and useful "I feel one ought to do simpler, cheaper things". If expensive, deep seismic studies were the object then the French–Chinese expedition would have been perfect to do the job, and would have done it well. But there was "no good gravity study" of the Tibetan plateau, for example, a lack that could be

Nature conservancy

UK unit lives hand to mouth

A REQUEST by the British Nature Conservancy Council (NCC) for extra funds to help restore the value of its research budget seems certain to be turned down by the UK Department of the Environment. NCC has asked for £200,000 towards its survey and monitoring programme, but the department has responded by suggesting that research is an area where particularly stringent savings are expected. NCC's grant from the department for next year, now under negotiation, will be announced later this month.

NCC's research programme has been steadily eroded since 1973, when the council was established as an executive body distinct from the Natural Environment Research Council. NCC research is now worth roughly half as much in real terms as in 1974. This is an untoward consequence of the Rothschild customer/contractor principle, by which some civil science research is commissioned by government departments. As, in recent years, the cost of salaries in NCC has, like that in other government departments, consistently outstripped the allowance made for it, the amount left over for research has declined. NCC also labours under an accounting peculiarity whereby it has to meet the cost of its staff pensions from its own funds.

Within a declining research budget, the amount NCC spends on research commissioned from the research councils has declined even faster. According to Sir Ronald Mason's investigation into government commissioned research, carried out for the Advisory Board for the Research

Councils (see *Nature* 306, 102; 1983), the amount spent with the Agricultural and Food Research Council and the Natural Environment Research Council fell from £1.3 million to £0.3 million in the five years to 1982–83 (1983 prices). One reason for the shift seems primarily to have been cost: NCC has found it more economical to employ young researchers on short-term contracts than to hire senior scientists who would often insist on working to a higher standard than was needed. The total annual cost of all NCC research is £1.5 million.

NCC now has to satisfy itself with a very pragmatic approach. According to its chief scientist, Dr Derek Ratcliffe, any possibility of commissioning basic research has long since been abandoned, and there is no chance of turning back the clock to the days when NCC's forerunner ran an experimental station of its own. All that NCC retains is a small group which spends most of its time providing site management advice to NCC local officers, and some survey teams. Most of NCC's resources are tied up in "renotifying" owners of Sites of Special Scientific Interest (SSSIs — see *Nature* 306, 525; 1983) and survey work aimed at identifying new SSSIs is a long way behind schedule.

Despite the gloomy outlook, NCC's chairman, Mr William Wilkinson, has been working hard to secure more research funds. It is felt that NCC needs hard factual information on sites to be able to make an authoritative case for their notification. **Tim Beardsley**

Molecular biology

UK to stay in Europe

BRITAIN'S Medical Research Council (MRC) has advised the Advisory Board for the Research Councils that the United Kingdom should adopt a whole-hearted commitment to the European Molecular Biology Laboratory (EMBL) in Heidelberg, West Germany. The news has come as a relief to EMBL, which has lived under the threat of a possible withdrawal of UK support ever since the Advisory Board for the Research Councils asked MRC to consider the benefits of UK participation in EMBL just over a year ago. The board will consider MRC's advice on 31 January.

The MRC view is based on a detailed assessment of the benefits and costs of UK participation in EMBL and the scientific merits and cost effectiveness of the research carried out there. The assessment, carried out by a group of five senior British scientists, concluded that the case for remaining in EMBL is strong on scientific grounds alone and remains strong even when costs are taken into account.

The group is not persuaded that the £1.2 million contributed by MRC to EMBL in 1983 (£1.5 million is due this year) would have purchased "an equivalent amount of work of equal quality" within the United Kingdom but does believe that EMBL's value for money should be increased.

The group recommends a more effective system of peer review and cost accounting for each of EMBL's scientific programmes but acknowledges that Professor Lennart Philipson has moved in the right direction since becoming director general of EMBL in 1982. It strongly approves of his new system of financial management of programme budgets, hopes and anticipates that costings will be linked to scientific reviews of the programmes but remains doubtful that the procedures for scientific review are adequate.

The problem, the group claims, is that the Scientific Advisory Committee of EMBL, which is charged with advising the laboratory council on EMBL's scientific programme, is in danger of being "seen as a creature of the director general" rather than offering independent advice to which the director general should be free to reply. Again, it welcomes Professor Philipson's moves to avoid such criticism but wants them further discussed and extended.

To judge the cost-effectiveness of EMBL, the MRC group chose to compare it with the MRC Laboratory of Molecular Biology (LMB) in Cambridge. The figures, summarized in the accompanying table, confirm suspicions that staff costs in Heidelberg are double those in Cambridge. The group considers that differential to be inevitable, given the salaries at comparable laboratories, notably the European Organization for Nuclear Research (CERN) near Geneva, and the allowances expected by expatriates on short-term contracts. It

also considers the greater recurrent costs at EMBL to be justified and points out that expenditure on consumables is equivalent in the two laboratories. The only way to cut costs, the group implies, would be to reduce staff — if stringent peer review revealed weaknesses.

The MRC group's view of the current scientific programmes at EMBL is that, like the curate's egg, it is good in parts. The cell biology programme excels and the out-station programmes at Institut Laue-Langevin, Grenoble and the DESY synchrotron in Hamburg are good. On the other hand, the recent investment in a differentiation programme is speculative and that on molecular structures is too large. Both instrumentation programmes are thought to be somewhat contrived to meet the original aim of EMBL to develop unique and expensive technology that few national laboratories would touch.

Costs at EMBL compared with LMB

	EMBL 1982 (£ thousands)	LMB 1982-83 per head
Staff costs		
Salaries	16.2	9.3
Allowances, insurance, etc.	5.5	2.4
Total	21.7	11.7
Extra costs		
Consumables	5.4	5.2
Recurrent	8.4	6.6
Total	32.9	18.1

Total EMBL expenditure for 1982 was £9.4 million of which 54.7 per cent was spent on the cost of 232 "man-years" of staff. Total LMB expenditure for 1982-83 was £4.6 million of which 47.3 per cent was spent on the cost of 186 "full-time equivalents" of staff.

Overall the MRC group argues that the benefits the United Kingdom receives from participating in EMBL outweigh those of withdrawing, even if there would be a short-term financial gain in doing so. Professor Philipson, who is generally pleased with the report, questions whether there would be even a short-term gain, once proper account was taken of the cost to the United Kingdom of redeploying many of the British nationals (about 20 per cent of the total) employed at EMBL. Mr D. Noble, MRC under-secretary, claims that the group's calculations did include that possibility.

Noble also said that MRC would be surprised if the Advisory Board for the Research Councils did not accept its recommendation to continue UK participation in EMBL. He hoped that the board would now see fit to advise the UK Government that its science budget should be adjusted to protect the MRC budget against losses at the expense of increased contributions to EMBL due to changes in the UK economy.

Peter Newmark

UK public health

Pathogens for suburbia?

Two laboratories at the UK Centre for Applied Microbiology and Research (CAMR) at Porton Down, in Wiltshire, England, may be closed in order to reduce costs. The two laboratories, the Special Pathogens Reference Laboratory and the Environmental Microbiology and Safety Reference Laboratory, carry out a varied programme of research in viral diagnosis, the production of vaccines and viral safety testing.

The proposal to close the laboratories is one of the options to be considered at a meeting of the Public Health Laboratory Service Board, on 26 January. Routine diagnostic work and possibly some safety work may be transferred from Porton to the new Central Public Health Laboratory building under construction at Colindale, in North London. Dr C. Gordon Smith, chairman of the Public Health Laboratory Service Board, says that a number of proposals will be discussed but stresses that none has yet been examined in detail and that no decisions have been made.

The news that diagnostic work might be moved to London nevertheless made front page news in the *The Standard* of London, under the banner headline GERM LAB PLAN FOR LONDON. Laboratory diagnosis of viral diseases, including Lassa fever and smallpox, has in fact been carried out at the existing Public Health Laboratory building at Colindale, which includes a high containment laboratory, since 1946. A statement issued by the Public Health Laboratory Service and the Department of Health and Social Security to calm the fears of Colindale residents says that "research involving highly dangerous organisms" will not be transferred to Colindale.

Among the staff of the threatened laboratories at Porton there is concern of a different kind. The work carried out there includes research on Ebola virus, Lassa fever and Marburg disease, and is aimed at reducing reliance on animals in diagnosis and producing vaccines. Botulinum toxins and anthrax and experimental whooping cough vaccines are also manufactured.

Porton staff hold that closure of the laboratories or partial transfer of some of the work to Colindale cannot be justified financially, and point out that experimental work is probably better carried out in a remote area.

It is far from certain whether the Health and Safety Executive would allow some of the work to be transferred to London. The staff at Porton say that they represent a national asset that becomes invaluable in an emergency and may spawn products of commercial value and complain that CAMR seems likely to be hit harder than other Public Health Service laboratories.

Tim Beardsley

US research computers

Agency expects funds to buy time

Washington

GRANTS from federal agencies allowing US researchers to purchase computer time are about to become respectable again. President Reagan's budget for 1985, to be published at the end of the month, will include substantial new funds with which the National Science Foundation (NSF) can help grant-holders to purchase time on supercomputers — machines such as the various models of CRAY computers. But the new funds will fail to satisfy at least two of the groups that have been urging that academic researchers should have more ready access to computer power.

One of these is the working group of the National Science Board (advisory to NSF) which last summer recommended that \$200 million should be spent over the next three years on ten supercomputer centres for university researchers. The new NSF programme will fall far short of that proposal, both in the amount of money available and because it is intended that the new funds should be used to buy time on existing machines.

Budget prospects

Dr Edward Knapp, director of the National Science Foundation, refused to comment last week on the other components of the NSF budget, but did say that he expected NSF to do as well as last year. The budget for the current fiscal year, published last February, gave NSF an increase of 18 per cent, the largest increase in a single year in the foundation's history.

Speaking on Monday at the National Academy of Sciences, however, **Dr George Keyworth**, the President's science adviser, did drop a hint about NSF's plant biology budget in the context of his general optimism about funding for agricultural science. Keyworth said that the plant biology programme at NSF had received a budget increase of 16 per cent last year, up to \$50 million. "I'd look for comparable growth again next year", he said.

Keyworth was not nearly so specific in discussing the competitive grants programme at the Department of Agriculture, although he did reveal that Secretary of Agriculture **John Block** had discussed the programme directly with the President and had received Reagan's personal support.

Even so, the new programme will mark a major shift of NSF policy on computing. Not since 1972 has NSF supported computer centres on campuses, while most federal agencies continue to balk at paying the full cost of purchased computer time under research grants. During the same period, this policy was abetted by researchers themselves, who preferred to buy their own mini- and microcomputers than to use central computing facilities

which, according to the working group, had fallen into disrepute.

Some of the consequences of that trend have been unfortunate. The National Science Board's working group concluded last summer that "important" research is not being tackled for lack of supercomputer facilities in the United States and cited the case of an astrophysicist who had found he could get access to the computing power he needed only by collaborating with a West German group. Under the new budget proposals, researchers will be able to buy time on CRAY and other computers such as those at Los Alamos and Livermore National Laboratories.

The new programme will nevertheless offend those who greeted the National Science Board's report with scepticism, saying that the supercomputer was a solution in search of a problem — and that the important applications of these machines are few. Meanwhile, an alternative policy is suggested by the work of a group of researchers at the California Institute of Technology (Caltech) who are developing what may prove to be cheap supercomputers. According to **Geoffrey Fox**, the theoretical physicist who directs the Caltech project, and **Charles Seitz**, a computer scientist, the few hours of computer time that a researcher may be able to buy with NSF funds will not add significantly to the computer power already at his or her disposal.

French research

Academics between millstones

RESEARCHERS in French universities who receive support from the Centre National de la Recherche Scientifique (CNRS), the principal research council, will soon have to "clarify" their position in relation to the university and CNRS. This follows an agreement last week between CNRS and the ministry of national education (MEN) which seeks to disentangle the sometimes complex relationships between research groups and their sponsors that have grown up through years of benign neglect, and a series of individual, idiosyncratic agreements between CNRS and individual groups.

Whether or not this will be seen to aid research depends the observer's point of view. It will certainly aid research management, and it will also aid university management — for in the past, university presidents have not always known what their own groups were doing. A new contract between a group and CNRS might commit the university to new overhead (providing laboratory space, for example) without the university having had any say in the matter.

On the other hand, however, the situation gave individual scientists the chance to

Fox says that "an IBM personal computer is more cost-effective than a CRAY" and that a few hours of CRAY time will not crack any problem that cannot be solved on a personal computer in a few hundred hours. What worries Fox is that under the new policy, supercomputer time "will be chopped up so that everyone will get a few hours. But you don't chop up Palomar into a pixel for each observer; I've never understood why we do that in computing."

The Caltech approach, according to Fox and Seitz, could give academics access to dedicated supercomputing power by cutting costs dramatically. They have already built a machine using 64 microprocessors of the kind used in personal computers with a power one-tenth of a CRAY machine but at only one-hundredth of the cost (about \$80,000).

The Caltech programming philosophy is also different. While CRAY machines have several processors that operate in parallel when the program-compiler identifies steps in a sequential program that can be executed simultaneously, the Caltech approach would parcel out steps in the solution of a problem to its separate processors. In the 64-chip version of the machine, each processor can communicate with six others and thus be kept running almost full-time.

At Caltech, the next step is to expand the desk-top 64-chip machine to a total of 1,024 chips. This would be equivalent to a CRAY machine in computing power but because of the use of mass-produced chips may cost only one per cent as much.

Stephen Budiansky

play off one part of the bureaucracy against another in the game of raising a grant. Under the new agreement, this will be harder, and there will be fewer corners where researchers not conforming to national policy can find rest.

The outline agreement describes "the ancient links, many and narrow" between CNRS and universities and claims that these justify "an effort of clarification and organization". The two sides have sought "precise texts" and "a common charter", which would be "legal, rational and balanced".

Under the charter, CNRS will draw up a general agreement with each university (or grand école), followed by a series of particular agreements with each group in that establishment, these particulars being placed in the context of the general agreement. The establishment as a whole will bear responsibility for applying the agreement, something that should give a university president more power in his (or her) own house (provided the president can control the new three-committee management structure of the university, a prospect which some French researchers find unlikely).

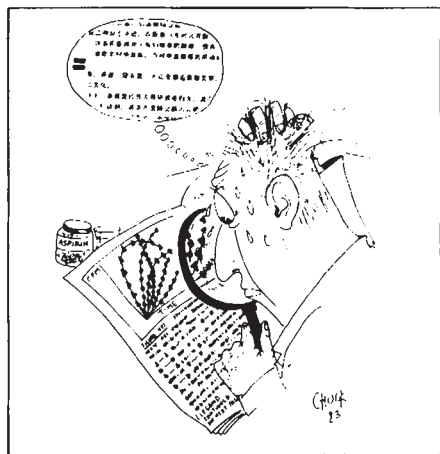
Robert Walgate

Making science a good read

SIR — The leading article "Must science be impenetrable?" (*Nature* 305, 477; 1983) concluded that little or nothing could be done to improve the situation, and that the authors are the most obvious culprits. I argue that the poor quality of illustrations contributes towards impenetrability, and that on this point the editors are the culprits.

The decipherment of a figure that contains more than one part can be very laborious, because the pertinent information is often buried in an overcrowded legend. This could be avoided if the following rules were applied:

- (1) When the graph includes more than one line, identify each by appropriate labelling.
- (2) When the figure contains several panels with the same abscissa and the same ordinate, usually identified as (a), (b) and so on, indicate on each panel its experimental specificity.
- (3) Whenever possible, use the same symbol for the same experimental condition in all comparable figures throughout the article.
- (4) When several symbols need to be used in a graph, use the open circle for the control condition; for instance, if an experiment has been performed without (○) and with (●) an addition or a treatment, use the symbols as indicated. (The open



circle is the simplest symbol available, and its "emptiness" suggests that nothing has been added.) Rule (4) should prevail over rule (3) when these two rules are in conflict.

(5) Make the labelling of abscissa and ordinate as precise as possible. For instance, it is preferable to write "Time after drug addition" than simply "Time". Also express results in moles rather than in c.p.m. or in absorbency units, sparing the reader the task of doing the conversion. If results are given in per cent, give the value of 100 per cent in standard units in the legend.

(6) The legend should be as short as possible, and could sometimes be restricted to the title since, ideally, the figure would be fully intelligible without making

reference to the legend.

From my own experience of the biochemical literature, I know that, if labelling of figures is still very limited, it is because it is systematically discouraged by subeditors, whose policy is to push all the information into the legends. With modern technology, the cost of lettering can no longer be an excuse for this negative attitude.

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Nicotine delivery

SIR — The concern of the British Royal College of Physicians (*Nature* 1 December 1983, p.418) with smoking and health is justified and well directed towards the immediate problem of reducing the number of people who may be influenced by cigarette advertising. The problem though, is that cigarette smoking is a means of drug delivery that is both crude and effective. If one assumes that some number of people in any society want to use drugs for a variety of reasons and that smoking is merely a way of delivering an addictive drug, the problem becomes conceptually simpler.

Addiction to the natural alkaloid nicotine is widespread and social tolerance of the drug and its users is such that for years a debate existed over whether chronic users were actually addicted in the same sense as opiate users. Semantic differences apart, smokers mostly smoke to deliver nicotine to their mucus membranes so it can be absorbed into the blood.

Alternative routes of drug administration more cosmetic than chewing tobacco or snuffs should be developed so that the nicotine addict has alternatives to cigarettes. Nicotine chewing gum has had limited success, but may soon become available worldwide. Another alternative might be transdermal application much in the manner of nitroglycerine and scopolamine patches. Nicotine "inhalers" might also be feasible if dosage could be adjusted.

I would appreciate receiving correspondence from individuals or groups actively engaged in finding alternative methods of nicotine delivery in humans.

CECIL H. FOX

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Prudent antibiotics

SIR — A leading article in *Nature* in 1981 (*Nature* 292, 661) referred to the publication of the "Statement Regarding Worldwide Antibiotic Misuse", which was signed by more than 200 physicians and

scientists and intended as an alert to the problem of antibiotic abuse.

The article raised the question of how the problem would continue to be addressed. The response generated by the statement enabled the Alliance for the Prudent Use of Antibiotics (APUA) to be established at the end of 1981. Its members include physicians, scientists, medical and public health personnel and members of the general public from more than 50 countries. The alliance has adopted a positive rather than a negative approach by advocating and defining good usage. Its activities include the gathering and dissemination of information and education on antibiotics and their use. The alliance has begun to organize a network of groups and individuals who are working in their own countries to improve antibiotic usage. The *APUA Newsletter* has opened a line of communication among the members. We recognize that it is at the level of the consumer — patient, physician, farmer, gardener, pet owner, beekeeper — that the rational use of antibiotics must be understood and accepted.

It is our long-range objective to implement changes in the use of antibiotics which will preserve their therapeutic effectiveness.

STUART B. LEVY

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Gripes of wrath

SIR — I read Walter Gratzer's review of Fowler's *Modern English Usage* (*Nature* 10 November 1983, p.134) and was both saddened and amused at the examples he quoted of uncouth English.

My own pet irritation is the use of 'in the order of' in place of 'of the order of'. This has become very common, but I do not recall ever coming across it more than twenty years or so ago. I believe it is an example of a phrase that "escaped" from scientific usage, was mangled by those who do not understand the meaning of order of magnitude, and then was hypnotically re-adopted by all because of the frequency of its appearance in the distorted form.

Other pet aversions of mine include "anticipate" when "expect" is meant (we anticipate completion by the end of the year), and the introduction of a quite inappropriate conditional qualification (symposium programmes are available in the foyer if delegates care to help themselves).

Finally, an ambiguity in a classified advertisement seen many years ago which never fails to amuse me when I recall it: Miss E. Brown, wardrobe dealer, having cast off clothing of all descriptions, invites inspection.

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Publishing "in conference"

SIR — I should like to draw attention to an alarming trend in the biological literature — the reporting of scientific data at conferences followed by publication of the abstracts in journals where they acquire the cachet of fully refereed papers.

During a recent literature search (using the BIOSIS 80 system) the key words "Ia antigen", "interferon", "macrophage" and "prostaglandin", in various combinations, elicited a total of 33 references for 1980-82 of which 19 were abstracts from conference proceedings. This struck me as being overbalanced. I do not question the validity of the data or the integrity of the investigators, but it is not possible to assess a piece of work from a 300-500 word abstract which, presumably, has not been refereed.

The two major "offenders" in my small sample were, coincidentally, both American, in particular, abstracts from the annual meeting of the Federation of American Societies for Experimental Biology published in *Federation Proceedings* and the annual meetings of the American Federation for Clinical Research published in *Clinical Research*. Such abstracts are subsequently extensively quoted in the literature and may be the only source for the work being discussed.

This trend seems to have come about because of the greatly increased numbers of scientists wishing to publish and the consequent increased pressure for space in conventional journals, the pressure on conference organizers to include all submitted abstracts and the increased use of poster sessions — impossible to referee. The overload of submissions is made worse by the apparent tendency of conference organizers to base their acceptance of registration on whether or not the applicant wishes to submit an abstract.

An example of these trends has recently been seen in the British Pharmacology Society, where data used to be presented as a talk and, following discussion, refereed "on site" by a show of hands from the members of the society. If the authors wished it the abstract was then published in the *British Journal of Pharmacology*. With the introduction of poster sessions and the consequent reduction in the possibilities for adequate reviewing, the society now publishes abstracts as a supplement to its journal — a laudable attempt to indicate the comparative lack of refereeing.

The trend towards publication of abstracts only is disquieting. A solution will not be easy to find, touching as it must the "publish or perish" syndrome. The simplest solution would be not to publish abstracts of meetings — perhaps a little drastic. Or the editors of the relevant journals could ask for a fuller abstract of the work presented to be adequately refereed before publication.

While it is not possible to blame scientists

(myself included) from taking this easy road to publication, the inclusion of unrefereed conference abstracts in subsequent articles where they acquire the same pedigree as fully detailed and refereed papers should be viewed with some unease as this practice is open to considerable abuse.

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● Perhaps data-banks should admit as search criteria Boolean specifications such as "not abstract". — Editor, *Nature*

India's bait

SIR — I sympathize with the Indian Government's attempt to lure expatriate scientists and doctors back home through programmes such as TOKTEN and through the proposal to create a sophisticated technological city (*Nature* 305, 350; 1983). But short-term solutions such as these are not the answers. India needs a coherent science policy and infrastructure to stem the flow of brain-drain and to lure the expatriates. As a first step, I suggest revitalization of the universities with up-to-date curricula facilities and competent indigenous and expatriate teachers.

I graduated from Kerala University in 1965 with a BSc in chemistry, but without having had the opportunity to use even a pH meter and without having been exposed to many of the fundamental principles of chemistry. When I joined the Training School of the Atomic Research Centre (Bombay) immediately afterwards I was confronted with advanced chemistry concepts (which at first went over my head), and a plethora of sophisticated instruments. I literally had to burn the midnight oil to bridge the almost inseparable gap between the university and the research centre.

I strongly urge the Indian authorities to rejuvenate science education in universities throughout India (not just in Bombay, Delhi or Madras). When the universities become centres of excellence, the expatriates will return home without the need for baits. One need only recall how Nalanda and Taxila, two excellent centres of learning in ancient India, attracted even foreign scholars to India.

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SIR — We would like to add a few of comments concerning the creation of a UN biotechnology centre and India's bait for expatriates (*Nature* 305, 350; 1983). We are of the opinion that many of the Indian scientists and doctors working abroad would like to go back to India. We would like to point that a recent survey by E.

Garfield (*Sci. Public. Policy* 10, 112-127; 1983) has shown that India is the "super-power" of Third World countries in scientific research.

The establishment of a biotechnology centre in India, either at New Delhi or Bangalore, would attract many of the Indian scientists working abroad to return to their country and promote science. For this reason, we urge UNIDO to reconsider India as a site for such a centre.

The announcement by the Prime Minister, Mrs Indira Gandhi, of the plan for a "technology city", where Indian scientists now working abroad would be able to work in conditions to which they have become accustomed, is a constructive step towards bringing scientists of Indian origin back to India. The return of meritorious scientists to India would create a competitive and productive environment for those already working in Indian universities and other research centres.

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Grains of truth

SIR — D.A. Williams claims in his letter (*Nature* 1 December 1983, p.420), that those who attended the Royal Astronomical Society discussion meeting on "Are interstellar grains bacteria?" were impressed by McDonnell's photographs of interplanetary dust in which silicate crystals were visible. He also claims that Hoyle conceded that interstellar bacteria are not alive. In point of fact Hoyle only said, in response to a question, that he had never claimed that they were alive, which is not quite the same thing.

Related to this point is the argument that bacteria would be photodissociated on scales of the order of 1,000 years. However, similar arguments were put forward by chemists to refute the suggestion that there were molecules in interstellar clouds — a position no longer held as shielding mechanisms ensure the long-term stability of organic interstellar molecules.

McDonnell's photographs do not show any evidence of the depletion of interstellar grains but only of the high velocity impacting interplanetary dust (micrometeorites), and were therefore irrelevant, as McDonnell suggested in his opening comments. Indeed the one thing that was held to be clearly disproved at the meeting was that the 10-micrometre feature was not due to siliceous material.

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Exotic nuclear decay discovered

The discovery, nearly a century after Becquerel, of a novel mode of radioactive decay is a surprise, but one that confirms α decay as the chief means by which heavy nuclei shed mass.

THOSE who decorate their offices with wall charts showing the isotopes, stable and otherwise, of the elements are in for trouble. As things are, these elaborate diagrams usually show by means of a colour scheme of some kind which radioactively unstable nuclei decay by which means. Decays in which the α - or β -particles (electrons) are emitted predominate, but the diagrams must also make room for other less common processes — positron emission, internal conversion (of an electron in an inner shell) and even fission. Now the same wall charts will have to accommodate radioactive decays in which unstable nuclides emit substantially heavier nuclear fragments than the familiar α -particle.

That is the burden of the discovery (see p.245) reported this week from the University of Oxford by H.J. Rose and G.A. Jones of a handful of unmistakable carbon nuclei among the decay products of radium-223 (^{223}Ra), hitherto marked in the charts as a simple α -emitter with a half-life of 11.2 days. What they have shown is that ^{223}Ra nuclei occasionally, but significantly, decay with the loss not of an α -particle but of a carbon-14 (^{14}C) fragment. Anthropomorphically, from the point of view of the nucleus so to speak, this novel arrangement has obvious advantages. The decay scheme of ^{223}Ra involves the successive emission of three α -particles (to give radon-219, polonium-215 and lead-211) followed by an electron, to give bismuth-211. (This decay scheme, which begins with uranium-235, predominantly leads to lead-207 by the further emission of two α -particles and an electron.) So why should not an unstable ^{223}Ra nucleus shorten this tortuous process by eliminating a substantial part of the charge and mass that must ultimately be lost in a single package? What now emerges is that that option is not as rigorously forsworn as the conventional wall charts suggest.

So how can it be that the best part of 90 years has passed since Becquerel's discovery of radioactivity before it has been recognized that α - and β -emission are not the whole of radioactivity? That is the obvious question that will now arise. The proper response, as a glance at the article by Rose and Jones will show, is that the discovery of ^{14}C emission from ^{223}Ra , important though it is, serves chiefly to emphasize that α -particle emission is the chief means by which radioactively unstable nuclei lose mass. Indeed, in the

observations now reported, there are roughly 1,000 million times as many α -particles and ^{14}C nuclei in the decay of ^{223}Ra , perhaps as vivid a proof as there could be of the dominance of the familiar mechanisms of decay.

The rarity of these events is also the explanation why this novel form of radioactivity has not previously been found. So much can be told from the account by Rose and Jones of their observations, which entailed more than half a year of running time with detectors arranged so as to distinguish between single ^{14}C fragments and more frequent occurrence of groups of three α -particles from the nearly simultaneous decay of separate ^{223}Ra nuclei. (The fact that one of the two detectors seems at one stage to have been put out of action by accumulated radiation damage is a telling measure of the numbers of α -particles involved, even if the fault appears to have been an ambition to avoid buying new material for this purpose.) In the circumstances, there is no reason to think that the discovery that ^{223}Ra can decay with the emission of a ^{14}C particle will quickly be followed by similar observations in respect of other nuclei. Rose and Jones say they are looking at another candidate nucleus, but there will be few groups with the stamina that compels competition.

Some, no doubt, will even ask whether this search for exotic and necessarily rare mechanisms of decay can be worth the trouble, now at least that it has been demonstrated that such mechanisms are not entirely excluded. The simple answer, as always, is that the neglect of phenomena on the simple grounds that they are only rare, or that their effects are small, is never justified. On this occasion, several important questions arise, not the least of which is why the first exotic fragment to have been found in the decay of a radioactive nucleus should have been the unstable ^{14}C nucleus and not the stable ^{12}C , itself simply a combination of three α -particles.

Superficially, the calculation of the rate of some specified radioactive decay is a simple problem in elementary quantum mechanics, often found in introductory textbooks. An α -particle, for example, can escape from an unstable nucleus only if it can tunnel through the potential barrier against disintegration caused by the nuclear forces that hold even unstable nuclei together. The chance that if such a

particle exists within the nucleus it will then escape, or the rate of the corresponding disintegration process, is thus a function of the height of the potential barrier, its width and of the total decrease of the potential energy of the system once the disintegration has taken place.

This calculation, first made in simple form by Gamow, has more recently been much refined and accounts for the "Gamow factors" used by Rose and Jones as part of their reason for believing that their disintegration products are ^{14}C and not some other isotope of carbon. But even on this simple picture, one huge uncertainty persists — the chance that a particle of the kind that eventually tunnels successfully through the barrier can be held, if only momentarily, to exist. What emerges from what Rose and Jones now say is that the "preformation probability" of ^{12}C would have to be comparable with that of the much simpler α -particle if it were to be produced in a disintegration. That cannot be the case, at least on this simple picture. It is relevant, but sobering, also to recall that the preformation probability of ^{14}C inferred from the results now reported is substantially greater than might have been expected from simple considerations, which is a reminder that the calculation of the absolute rates of even α decay, for example, is still not feasible.

What, in these circumstances, may be made of the discovery now reported? If, in the years ahead, there should accumulate a handful of examples of exotic kinds of radioactive decay in unstable heavy nuclei, it should at least be possible to glean some empirical information on the chance that various groups of nucleons will assemble into different potential disintegration fragments in neutron-rich heavy isotopes such as ^{223}Ra .

Hopes that exotic disintegration routes may have some practical value in other fields, perhaps in some novel technique of geochronology, seem, however, to be slim. Exotic disintegrations of the kind now identified are simply too few compared with the more familiar α disintegrations for their daughter nuclei and their products to be recognizable among the end products of the three principal radioactive series. (And ^{14}C is in any case unstable.) But at this stage it would be rash to rule out such possibilities. Too many confident predictions that new phenomena have no practical significance have too often been falsified for that course to be wise. **John Maddox**

Marine geology

Mapping the sea floor by satellite

from Roger N. Anderson

WHAT may well become one of the most famous satellites ever launched into Earth orbit transmitted for only three months before an electrical short-circuit killed it dead. Clearly, it will not go down in history as one of NASA's most successful engineering exploits. So how is it that the satellite may yet become a scientific legend?

Launched in 1978, with instruments primarily intended to map sea surface conditions, SEASAT also carried an ultra-sensitive altimeter. A continuous monitoring of the flight path of the satellite and the microwave travel time from the satellite to the sea surface and back again, allows the construction of an extremely accurate map of the topography of the sea surface. Much more than just temporal variability of the ocean surface (itself of importance) appears in such a map. For example, the map shows that the sea surface off the UK is several metres lower than the sea level off Iceland. In the Indian Ocean south of India is a huge trough — travelling from Africa eastwards towards Australia, a ship will sink over 100 m downwards and then gradually rise again. Why does this occur? Because excess mass beneath Africa and Australia pulls water away from the central Indian Ocean. Quite literally, the pull of the Earth's gravity is larger towards the edges of the Indian Ocean than at its centre. By mapping the sea surface height, one can map the Earth's gravity field since the ocean will always form a surface that is everywhere perpendicular to the direction of the gravitational pull.

If a satellite flies over a piece of ocean day after day, constantly mapping sea surface height, short-term disturbances will be averaged out and an accurate map of the Earth's gravity field can be generated. But why is such a map important?

For marine geologists the answer can be seen by considering, for example, a remote portion of the South Pacific Ocean west of the southern tip of South America and east of New Zealand (see Fig. 1). In over one hundred years of oceanographic exploration, actual depth measurements to the sea floor have been made only along the widely spaced ship tracks shown at the bottom of the figure. In only three months, SEASAT covered the same area with the satellite passes shown at the top of the figure. Obviously, if we could know how to transform gravity into bathymetry, SEASAT could make existing maps of the sea floor obsolete.

But there are military implications of such a data set too. Missile-equipped submarines, like the Trident, play a vital part in nuclear defence because they could survive a first strike. But submerged

submarines must navigate. Of course, they can float radio antennae to the surface to communicate with satellites to determine their position, but how long would satellites last if a nuclear war began? Just as Cruise missiles and Backfire bombers navigate at extremely low altitudes by following the terrain of the continents, submarines could navigate relative to the terrain of the ocean floor — if they knew that topography well enough. A map from SEASAT could be as good as a road map to a submarine commander.

How then to recover the maximum amount of information from the SEASAT altimeter data? The Goddard Space Center, NOAA's National Ocean Service in Maryland, the US Navy and Jet Propulsion Laboratory in California have all had a crack at it. At low latitudes, satellite tracks are as much as 200 km apart — wider than the dimensions of geological features that SEASAT was capable of detecting. The difficulty is to preserve as much information as possible so that accurate maps of the gravity field and sea floor can be generated. Good maps were produced by these organizations (see, for example, refs 1–4), but by sacrificing details, through smoothing the data. With release of the SEASAT data to the academic community new techniques for extracting fine detail were developed. At the bottom of Figure 2 is shown existing bathymetric knowledge of an area in the southern Pacific. At the top is a gravity map produced by William F. Haxby of Lamont-Doherty Geological Observatory. The complete world-wide map is shown in reference 5.

What did Haxby do that those at other laboratories didn't? The secret is that the major geological features of the sea floor are strongly lineated as a consequence of plate tectonic processes. Trends identified in the SEASAT data were used to fill in the gaps in data coverage. The information derived from SEASAT was further enhanced by computing variations in gravitational force from a grid of sea surface heights. Think of driving slowly up a long grade to a precipitous cliff, then plunging down steeply into a valley, then up another long hill. Topography would record the gentle slope up, then the plunge down and the next slope, but the gradient of topography would record gentle slopes as virtually flat (not much change in topography from point-to-point) while cliffs show-up very dramatically because they are surrounded by a flat background, rather than by a 'real background'. Haxby went on to use north-south and east-west gradients of sea surface heights to calculate

the actual gravity anomaly by combining them using La Place's equation. The map Haxby produced highlights important features of ocean topography in a way that has never before been achieved.

In the example area alone two important discoveries were made⁶. First, the clear outline of fracture zones, even under sedimentary cover, could be made out. These are scars left in oceanic plates by past plate motions and can be used to map the past movements of the Pacific and Antarctic lithospheric plates. Second, seamounts or undersea volcanoes were discovered in places where no previous seamounts were known. Chains of such volcanoes denote locales on the sea floor where extended volcanic activity has occurred long ago in the geological past. Such 'hot spot' trails not only tell of past motions between plates, but of absolute motions of the plates relative to the ultimate source of the heat, the Earth's mantle.

In the gravity map not only can we see the sigmoidal fracture zones that show gentle shifts in motion between the Antarctic and Pacific Plates over geological time, but also a north-south topographical scar left by a several hundred kilometre jump of the East Pacific Rise to the west 50 million years ago (far right, centre of image).

But there is political danger in scientific success. In October 1984, GEOSAT will be launched to map further the variability of the sea surface. But for the first 18 months of its existence, all data it transmits will be classified. The US Navy states that after 18 months of mission-oriented science, they may operate a scientific effort for the satellite, if it is still working.

In response to strong scientific interest in such satellites, from not only marine geologists, but physical oceanographers as well, there are a number of planning efforts now going forwards in the US within the Federal Agencies, the National Academy of Sciences, and in the academic community coordinated by Joint Oceanographic Institutions, Inc. The efforts are focused on the opportunities for global scientific programmes offered by the new satellite technology and on the challenges presented to all these groups in learning to work together with this new kind of data. The hope is that science will gain a respected place alongside the military in the satellite exploration of our planet — and just as important, that academic scientists will get their hands on the raw data as quickly as those from the government laboratories. □

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Fig.1 Top: SEASAT orbital tracks for the region of interest. Residual geoid heights are shown along descending tracks (half the total data set). Bottom: distribution of ship tracks in the region for which digital bathymetric data exist. Less than half of these cruises employed satellite navigation (from ref.6).

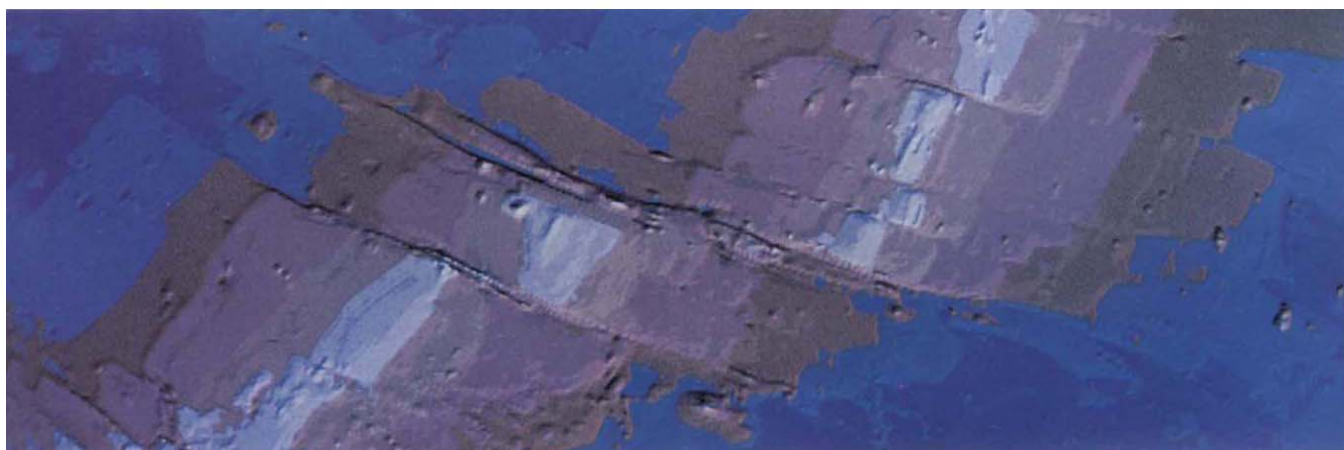
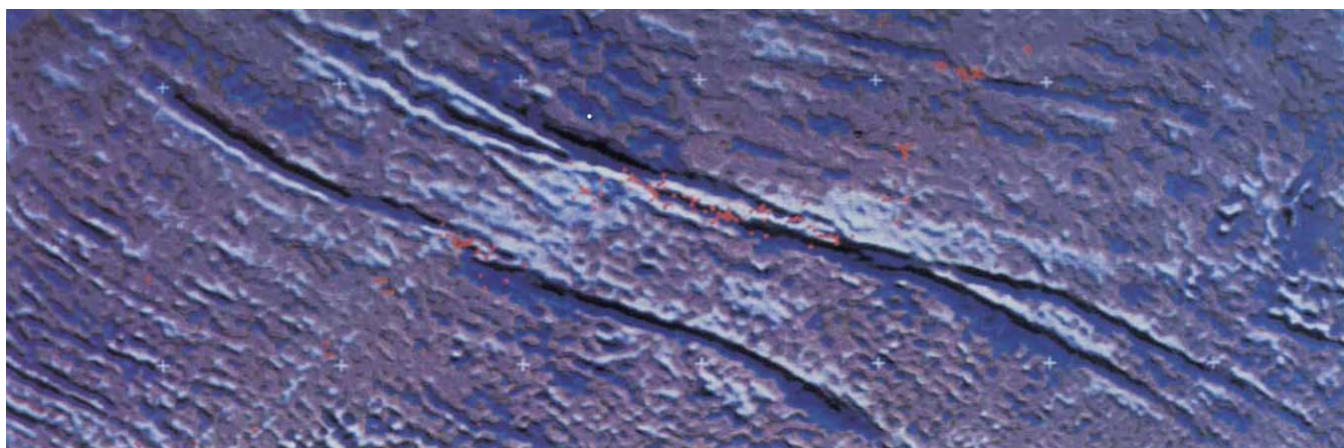
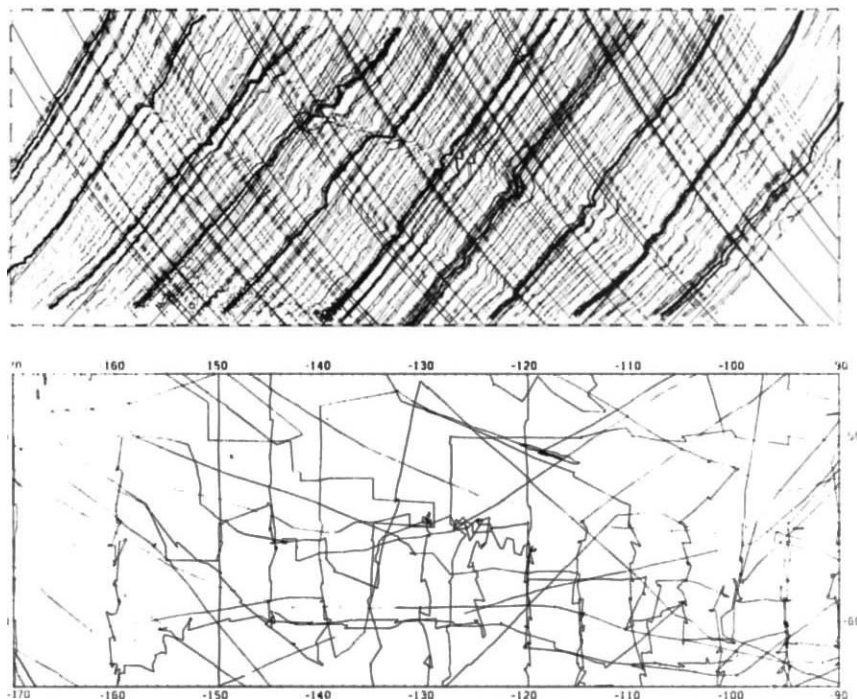


Fig.2 Top: colour relief image of gridded SEASAT-derived gravity anomalies in the region of the southern Pacific Ocean between 170°W, 90°W, 63°S and 46°S, contoured at 4 mgal intervals and illuminated from the NE. The gravity signatures of the major fracture zones (from north to south: Menard, Heezen, Tharp and Udintsev) are well defined as are several smaller fracture zones. Every 10° of latitude and longitude is denoted by a small white cross. The small red dots are epicentres of shallow earthquakes (WWSSN/NOAA sources, depths 0–70 km, $m_b > 4.5$) which occurred between 1961 and 1981. Bottom: colour image of gridded bathymetry in the same region, contoured at 500 m intervals and illuminated from the SE. The digital bathymetric data set for the Southern Hemisphere was prepared at Bay St Louis, Mississippi by scientists of the US Naval Oceanographic Office (from ref.6).

Astrophysics

The origin of gas in quasars — reverse stellar evolution?

from Martin Gaskell

WHAT is going to happen to a star situated next to the central energy source of a quasar as it is blasted by an intense stream of ultraviolet, X-ray and γ -ray radiation? A recent study by W.G. Mathews¹ makes the bold prediction that stars in such an environment will reverse the direction of their evolution!

One of the defining features of quasars is the presence of rapidly moving ($v \geq 10^4$ km s⁻¹) gas clouds with densities greater than 10^8 particles per cm³ (much denser than normal gaseous nebulae seen around hot stars). Because they produce wide Doppler-broadened emission lines these clouds are called 'broad-line' clouds and the region from which they come is called the 'broad-line' region. From considerations of photoionization equilibrium we can work out the distance of these broad-line clouds from the central energy source (the mean ionization depends on the intensity of ionizing photons and the rate of recombinations which depends on the density). This distance is about a light year for an average quasar. In Seyfert galaxies (a kind of low-luminosity quasar) the broad-line region is expected to be an order of magnitude smaller (light-weeks to light-months) and emission-line variability observations have independently confirmed this size scale². We are very confident, therefore, that the broad-line region is extremely close to the central energy source of the quasar.

The origin of the broad-line clouds is one of the major mysteries of quasars. The gas has a relatively high density, intermediate between normal gaseous nebulae and the density of the outer layers of stars³, and a chemical composition not very dissimilar from stars such as our Sun⁴, but these facts tell us very little. It is the motions of the clouds that are potentially the most important indicators of their origin. In the past all possible kinematic conditions have been given serious consideration — rotation in a disc, random orbits, infall and outflow — but it is the radiative acceleration theory that has been worked out in the greatest physical detail⁵⁻⁸. Although the issue is by no means completely settled there is mounting evidence that the broad-line clouds are indeed preferentially outflowing (rather than orbiting or infalling). For example it has been known for many years (from optical absorption lines) that ionized gas is outflowing from many high-luminosity quasars and even Seyfert galaxies⁹. Recently discovered systematic differences in broad-line profiles¹⁰ imply that at least the highest-ionization broad-line clouds are outflowing. Perhaps the

most direct evidence in favour of the radiative acceleration model is the correlation between line widths and physical conditions of broad-line clouds¹¹. This is readily explicable in radiative acceleration models (since the same radiation which is accelerating the clouds is also photoionizing them) but is hard to understand in models where gravity dominates the dynamics.

Despite the growing evidence in favour of the radiative acceleration model and the unsatisfactory state of alternative gravity-dominated models¹² the problem of the actual origin of the clouds is perhaps more acute than ever. In the radiative acceleration theory a relatively steady volume source of cloud material is essential since it is only gas starting outside some critical radius which is accelerated outwards. Gas clouds created inside that radius (for example, from tidal disruption of stars near the black hole or from the accretion disc) will fall inwards (this is in fact highly desirable since one needs an influx of matter to power the quasar). The stars within a light year or so of the central energy source are the most natural source of the broad-line clouds but it has been realized for some time that normal stellar mass-loss processes (for example, stellar winds) fail by an order of magnitude or two to produce the necessary mass outflow rates if the broad-line clouds are all being expelled from the nucleus. This has led some workers to consider whether the intense radiation of quasars can itself enhance stellar mass loss. An attractive possibility is pushing the gas off the surfaces of the many lower main-sequence red dwarf stars in the galactic nuclei by the same quasar radiation pressure that will accelerate it to high velocities¹³. While this can work for red giant stars, the surface gravities of red dwarfs turn out to be prohibitively high for this sort of radiative ablation and red giants are expected to be much too rare to provide enough mass.

Mathews¹ offers a startling way out of these difficulties by proposing that red dwarf stars close to a quasar have their internal structures changed by the quasar radiation and effectively reverse their evolution by expanding. These 'inflated' stars would have lower surface gravities and could be ablated by radiation pressure. The key fact Mathews points out is that low-mass stars in the broad-line region actually have more radiant energy incident upon them than they produce by nuclear reactions or gravitational contraction. Mathews argues that if more than a few per cent of this energy can be mixed through-

out the star a new configuration will be reached with an expanded star having a lower central temperature. Low-mass stars would then evolve in the opposite direction to their original contraction, towards the main sequence (hence the term 'reverse stellar evolution'). The hard X rays can deposit their energy relatively deeply in the stars and from there it can be mixed throughout the star either by convection or by large-scale meridional circulation currents (analogous to the situation in Jupiter where the incident solar radiation could do the same thing). Once the star has been inflated (or rather re-inflated) the radiative ablation process can remove matter efficiently from the surface of the star. After some 10^9 years or so the entire mass of the red dwarf star will have been removed. When the quasar has exhausted its supply of red dwarfs its activity will die down. This is in agreement with observations which show that quasars were far more active 10^{10} years ago when galaxies were young than they are now. Mathews even goes so far as to suggest that a possible deficiency of red dwarf stars in the inner one light year of our own galactic nucleus¹⁴ could be a result of past quasar activity.

The inflation/ablation model has a number of attractive points: it gives the needed enhanced stellar mass-loss rates; the distance from the X-ray source within which stellar inflation is possible is consistent with the size of the broad-line region inferred from completely different arguments; and the gas is automatically produced at the right density and temperature. Inflated stars are also more liable to collide and produce the hot 10^8 K intra-cloud medium. The question of whether the inflation mechanism does actually work will, however, require more detailed studies to settle it. It is certain that a star next to a quasar cannot have a normal cool 3,000 K surface, but just how the harsh incident X-ray flux will affect its internal structure is a very difficult thermodynamic problem depending on poorly understood theory. If the inflation process fails and some other adequate mass-loss mechanism cannot be found then the physical model for the quasar that has emerged in recent years will need radical modification. □

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Acquired immunodeficiency syndrome

The AIDS epidemic: continental drift

from Jerome E. Groopman and Paul A. Volberding

WHERE now to dig for clues to the aetiology and pathogenesis of acquired immunodeficiency syndrome (AIDS) is the major decision facing investigators this year. The study of AIDS has progressed rapidly since the initial recognition of the disorder in 1981 (ref. 1); largely through investigation of cases belonging to accessible high-risk groups such as homosexual men, intravenous drug users and haemophiliacs. The majority of those studied have resided in cities like New York, San Francisco and Los Angeles, the epicentres of the epidemic. But the cause of AIDS has not yet been identified, and digging in geographical areas outside these epicentres and investigation of atypical cases is probably going to be required.

Three types of case are now being most carefully studied: those outside the continental United States; those among persons with no known risk factors; and those that do not fit the original surveillance definition of AIDS of the Centers for Disease Control but are likely to result from the same causative agent(s).

There are currently nearly 300 cases of AIDS (defined as opportunistic infections and/or Kaposi's sarcoma in patients with unexplained cellular immunodeficiency²) in Europe. What distinguishes the European cases from those in North America is an intriguing link to Central Africa, particularly Zaire. Nearly all the 40 Belgian cases have had occupational or sexual contact with persons from Zaire or Chad.

A recent visit to Kinshasa, capital city of Zaire, by a team from the Centers for Disease Control and the National Institutes of Health has confirmed the existence of substantial numbers of AIDS cases in urban Zairian hospitals. Although it is still unknown whether AIDS in Central Africa predated the emergence of the disorder in the United States in the late 1970s, the occurrence of the disease outside the continental United States may provide important clues to not only its origin, but also its patterns of spread. During the past decade there appears to have been continental drift of the 'AIDS agent' among Africa, North America and Europe.

Among African cases in Africa and Europe there are about equal numbers of males and females; homosexuality does not appear to be a factor. AIDS may be transmitted among Central Africans through pathways including heterosexual sexual contact and medicinal use of unsterile needles.

The pattern of occurrence of AIDS among Haitians in the United States and in

Haiti is more controversial^{3,4}. There are claims that it is explicable by covert male homosexuality and that the taboos against homosexuality are so strong in Haitian society that accurate epidemiological information could not be obtained at first. It is important to resolve this controversy and to resolve it in a fashion that avoids any possible stigmatization of a socially vulnerable group like the Haitians and that does not bow to political pressures.

Studies of Central Africans and Haitians are now being carried out with special attention being paid to possible aetiological agents of AIDS. The current leading candidate is the human T-cell leukaemia virus (HTLV)⁵⁻⁷. Isolates of HTLV-like viruses have been obtained by both American and European groups from peripheral blood, lymph nodes and spleen, and molecular mapping of the isolates may point to genomic regions important in the pathogenesis of the immune suppression. Despite such endeavours, the prevalence of a number of other viruses in both Africa and the Caribbean may confound rapid identification of a unique aetiological agent.

The situation will probably be very different among persons in the US who have contracted AIDS but have experienced no known risks other than exposure to blood products. Such previously healthy persons do not have lifestyles or travel histories that particularly predispose them to infections with viruses such as cytomegalovirus, Epstein-Barr virus or HTLV. There are currently 38 cases (30 adult, 8 children) of AIDS in the United States that appear to be associated with blood transfusion.

The earliest case of transfusion-associated AIDS occurred in mid-1982 and there has been a rapid increase in cases since that time⁸. The incubation time (time from transfusion to onset of clinical symptoms) of AIDS in such cases ranges from 10 to 45 months in adults with a median of 25 months. Individuals have developed AIDS after blood transfusion with as few as two units of blood but most cases have had multiple units from several donors.

In nearly all the cases studied to date, the Centers for Disease Control was able to identify at least one donor who was 'suspicious' in that he was a member of a high-risk group for AIDS and often had associated immunological abnormalities. It is intriguing that only one 'suspicious' donor actually had AIDS as defined by the Centers for Disease Control while the others had lymphadenopathy, constitutional symptoms, or were entirely

asymptomatic. This may have important implications in identifying who is capable of transmitting the disorder, at least through blood donation. There may well be an asymptomatic carrier state of the syndrome analogous to that of hepatitis B. In addition, infection with the putative AIDS agent may be transient while immune injury persists, eventually leading to clinically apparent disease. If this is true, identification of the causative agent, which may be present for only a short period of time, will require large prospective studies of individuals likely to be exposed to the agent and aggressive serial procurement of tissue.

The extraordinarily long incubation period apparent from these transfusion-related cases may be due to unknown host susceptibility factors or to a 'slow virus'. It is of note that the majority of transfusion-related AIDS cases have occurred in those regions in which AIDS is quite prevalent (New York and California) while cases among haemophiliacs does not follow the same geographical pattern. This probably relates to the fact that the factor VIII concentrates used by haemophiliacs are distributed nationwide while more standard blood products are locally distributed. AIDS has now become the second major cause of death (after haemorrhage) in haemophiliacs in the United States.

It is clear that the original surveillance definition of AIDS developed by the Centers for Disease Control was purposely narrow in scope and has excluded from the data base large numbers of individuals who probably have a milder form of the disorder. AIDS-related complex (ARC) is a constellation of clinical findings including unexplained generalized lymphadenopathy, fever of unknown origin, night sweats, weight loss and chronic diarrhoea with associated laboratory abnormalities indicating impairment of the cellular immune system. ARC is occurring in the same groups at high risk for AIDS. Data are just being accumulated on ARC patients, but it appears that their syndrome is transmissible in the same manner as AIDS and is probably caused by the same agent^{9,10}. It is estimated that there are 5-10 times as many persons with ARC as AIDS in the United States, or about 15,000-30,000 cases taking CDC statistics on AIDS.

The incidence and prevalence of AIDS in certain areas of the United States is now beginning to be determined. A cohort of 6,500 homosexually active men in San

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Francisco has been followed since 1978 as part of an epidemiological study of hepatitis B. The overall prevalence of AIDS in this group is one per cent and, for a subset of men 30–40 years old, nearly two per cent. The yearly incidence of AIDS in this cohort is about 0.5 per cent. This is a remarkably high incidence and prevalence for a communicable disease which is usually fatal. It can be estimated that about

5–10 per cent of this cohort may develop ARC. Should these numbers be accurate, then the United States is facing an epidemic of significant dimension and measures should be taken to contain it. □

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Cell biology

Gene amplification and drug resistance

from Margaret Fox

SINCE the original reports in 1978 by Schimke and co-workers of the association of increased dihydrofolate reductase (DHFR) gene copy number with overproduction of DHFR protein and resistance to the drug methotrexate (MTX) many other examples of the association of gene amplification with drug resistance have been documented. Leading workers in the field were recently brought together at a symposium* where the major problems of the relationship between gene amplification and the development of drug resistance were discussed.

At the symposium it was made clear (G. Stark, ICRF, London and G. Wahl, Salk Institute, San Diego) that gene amplification is perhaps something of a misnomer because in almost all cases seen to date varying amounts of flanking DNA sequences are co-amplified with the selected gene to give an amplified unit which can vary from approximately 40 kilobases (kb) to over 1,000 kb in different cell lines selected for resistance to the same drug. The replacement of the term 'gene amplification' by that of 'DNA amplification' was proposed.

Most studies have been made on a variety of *in vitro* cell lines derived from human tumours, and 'normal' and transformed rodent cell lines. It is clear that if an appropriate enzyme inhibitor which binds tightly is available, then cultured cell lines will respond by amplifying the gene coding for the target protein together with varying lengths of flanking sequences. The rate and degree to which amplification occurs is directly proportional to the concentration of the inhibitor used in the case of resistance to N-(phosphonoacetyl)-L-aspartate (PALA) in BKH cells (Stark).

Data from other investigators (R. Kellems, Baylor College of Medicine, Houston and R. Wilson, Institute of Genetics, Glasgow) indicated that the level of protein overproduction was almost unlimited given sufficient selective

pressure. R. Kellems, for example, reported that adenosine deaminase (ADA) levels could be increased over 10,000-fold — production then amounting to 50 per cent of total soluble protein — in cells grown in adenosine, alanosine and uridine in the presence of deoxycorformycin. Similarly, very high levels (approximately 20 per cent of total cellular protein) of glutamine synthetase were achieved by selecting cells for resistance to the inhibitor methionine sulfoximide (Wilson). In the case of ADA, overproduction could be totally accounted for by a corresponding increase in the abundance of mRNA and ADA-coding DNA sequences which were carried on multiple double minute chromosomes.

In other drug-resistant cell lines the increase in gene copy number differed considerably, ranging from a five-copy increase associated with a very large homogeneously staining region (HSR) in a MTX-resistant HeLa cell line (J.M. Trent, University of Arizona) to the production of 600–1,200 copies of DHFR genes in EL4 mouse cells and PG19 melanoma cells (C. Bostock, Mammalian Genome Unit, Edinburgh) and a 200-fold increase in CAD sequences in PALA-resistant lines (Stark).

Sequences flanking the selected gene have always been found to be co-amplified, but the size of the co-amplified DNA varies greatly from one cell line to another. It is not clear what determines the size of the amplified unit but the co-amplified DNA sometimes expresses novel proteins which can be detected on SDS polyacrylamide gel electrophoresis. In cells selected for overproduction of adenylyl deaminase (AMP. D) polypeptides other than AMP.D are overproduced (M. Debitasse-Buttin, Institute Pasteur, Paris). Analysis of several clones suggests that different polypeptides are overproduced to different extents in different clonal isolates. The implication is that the co-amplified DNA has undergone a variety of recombination events.

Overproduction of glutamine synthetase and phosphoribosyl pyrophosphate amido transferase was reported in some cell lines

selected for overproduction of asparagine synthetase by the use of β -aspartyl hydroxamate and albizzin (I. Andrulis, Hospital for Sick Children, Toronto). In these resistant cell lines a good correlation was found between the level of DNA sequence amplification and abundance of mRNA and protein. Considerable differences in the levels of overproduction of asparagine synthetase achieved were observed in resistant cells selected in the two drugs. Multistep selection in albizzin resulted in 260-fold overproduction of normal enzyme but with β -aspartyl hydroxamine prolonged selection resulted in the accumulation of an apparently mutant enzyme.

In PALA-resistant clones which overproduce the CAD protein (Stark), a 45 kb sequence containing the 24 kb CAD gene was always amplified and the co-amplified DNA also coded for three other RNAs which were overproduced to different extents in different mutants. Overproduction of the multifunctional protein UMP synthetase which is inhibited by pyrazofurin and increases 40-fold in response to selection was reported by P. Suttle (University of Texas, San Antonio). These data indicate that there is not always a direct quantitative relationship between the level of gene amplification and the level of mRNA abundance.

The facility with which genes can amplify in different cell lines and the differences between cell lines with respect to whether the amplification events result in the formation of double minute chromosomes, very large homogeneously staining regions, or abnormally banding regions were much discussed. In human tumour cells and in *in vitro* cultured mouse lines, both double minute chromosomes and homogeneously staining regions have been reported, the former being generally associated with more unstable phenotypes. Amplified sequences contained in homogeneously staining regions can also be slowly lost on prolonged growth in the absence of selection (J. Biedler, Sloan-Kettering Institute, New York) but abnormally banding regions seen mostly in mouse cells appear stable. In Chinese hamster cells double minute chromosomes are rarely if ever observed. There were also suggestions of a tighter control of amplification in differentiated cells (F. Fougere Destrachette and M. Weiss, National Centre for Scientific Research, Gif-sur-Yvette).

The question of permissiveness for amplification was discussed by J.A. Wright (University of Manitoba) who reported that isolation of ribonucleotide-reductase-amplified cell lines from human diploid fibroblasts was much more difficult than from permanent rodent cell lines. Human tumour cells *in vivo* and cell lines derived from human tumour cells appear to amplify their genes with relative ease (Trent).

Several contributors addressed the

*The 18th Paterson Symposium was held at the Paterson Laboratories, Manchester, on 17–19 October 1983.

question of the structure of the amplified unit. A model involving translocation and homologous recombination was suggested by W. Flintoff (Health Sciences Center, London, Ontario); Bostock and Stark also discussed several possible models. Considerable evidence in favour of recombination being involved in the generation of amplified DHFR and CAD units was presented by both these authors. Amplified sequences have been assayed for novel fragments (joints) after differential hybridization of control and amplified DNA and subsequent cloning of amplified DNA in λ vectors. In all amplified lines novel sequences have been detected, many of which are unique to individual amplified clones. Stark posed the question as to whether selection procedures act on previously amplified sequences or whether amplification is generated by the selective agent. Data from fluctuation analysis indicated that amplification by the selective agent is of only minor importance in comparison with the action of selection on previously amplified sequences.

The question of whether genes amplify *in situ* or whether translocation and rearrangement are involved was addressed by Wahl. Cloned CAD genes of Syrian hamster were introduced into Chinese hamster cells where they can be recognized because they possess unique sequences. The donated genes were shown by *in situ* hybridization to reside on different chromosomes to the one containing the native gene and, in one case, they amplified spontaneously at a frequency at least 100-fold higher than that seen in three other transformants or wild-type cells. The suggestion is that flanking chromosomal sequences mediate the amplification event although it is conceivable that the hyper-amplifiable structure was created by the insertion of donor genes, for example, by disruption of a replication origin.

Several major questions remain unanswered. First, are normal diploid cells capable of amplifying their genes, in particular do human diploid cells possess this capacity? Human tumour cells appear to be amplification proficient, but is this an initial event in the generation of the malignant phenotype related to overall loss of genomic control or do specific alterations in DNA sequences occur followed by a cascade of events? How frequently is DNA amplification involved in intrinsic and acquired resistance in human tumours *in vivo*? Suggestions that amplification is directly involved in the generation of drug resistance come from studies of oat cell carcinoma with the *c-myc* probe in which some correlation between the presence of amplified *c-myc* sequences and refractoriness to chemotherapy was evident (Trent). Do drugs used in chemotherapy actually trigger amplification and could we prevent it if we knew the mechanism? □

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Virology

Viruses and tissue injury

from Bernard N. Fields

IN A report in this week's *Nature*, Oldstone and colleagues (p.278) describe how infection of young mice with lymphocytic choriomeningitis virus leads to changes in growth and glucose regulation¹. What is remarkable about these findings is that there is no histological evidence of tissue injury in the pituitary glands of the infected mice — even though growth hormone deficiency was clearly detectable, transplantation of growth hormone-producing cells allowed infected mice to grow normally, and the virus was shown to be replicating in the pituitary. The observation makes clear that injury as detected by conventional pathological analysis may be absent in the face of profound functional impairment and should stimulate a closer look for viruses in other diseases where the function of differentiated cells is abnormal.

The work of Oldstone and colleagues is one of a number of recent reports demonstrating that viruses are capable of injuring mammalian hosts in a variety of ways not previously suspected.

Originally, it was thought that viruses were the cause of acute or occasionally chronic infections associated with detectable injury to tissues. With the development of cell culture techniques, particularly in the 1950s, viruses were detected and often identified by their characteristic cytopathic effects. The extraordinary diversity of the behaviour of viruses was further revealed by unusual or exotic occurrences such as that of subacute sclerosing panencephalitis (a progressive fatal neurological illness) occurring years after measles infection² and the identification of the exotic spongiform encephalopathies (such as Kuru of man and scrapie of sheep) in which a progressive degenerative neurological illness occurs without fever or an inflammatory reaction and is due to a transmissible agent that has a number of extraordinary properties (possibly including the absence of nucleic acids)³. Recent studies using animal models have expanded further the possible role of viruses in diseases of unknown aetiology and illustrated new ways that viral injury may be mediated. It is now clear from experiments in mice that viruses can specifically injure endocrine tissue, leading to abnormalities of endocrine secretions⁴, and the work reported in this issue shows that lymphocytic choriomeningitis virus can alter pituitary cells, causing them to produce hormonal deficiencies, while producing no detectable cytopathic effects⁵.

It is especially interesting that the findings with lymphocytic choriomeningitis virus demonstrate how endocrine

tissue may be affected since viral infections have long been considered as leading candidates in the causation of insulin-dependent diabetes mellitus⁶. While there are a number of important differences between insulin-dependent diabetes mellitus and the lymphocytic choriomeningitis disease described by Oldstone and colleagues (particularly the presence of tissue injury as manifested by insulinitis in the former), insulin-dependent diabetes mellitus serves to illustrate additional ways by which virus infections may result in disease. In particular, the studies of Notkins and co-workers have shown that certain viruses may directly infect pancreatic β cells in mice resulting in chemical diabetes⁷. Viral infections of mice may also result in autoantibodies to growth hormone and a polyendocrinopathy⁴. While the relevance of these mouse models to human illness has not been proven, it is clear that antihormone antibodies and antitissue antibodies are additional important ways that viral injury may be mediated.

Evidence for another route by which virus infection may lead to the production of autoantibodies and tissue destruction has been seen in recent studies of the mammalian reoviruses⁸. Viral receptors were postulated to share structural features (idiotypes) with antibodies directed against the viral haemagglutinin (the protein of the virus responsible for attachment to the cell). Anti-idiotypic antibodies directed against antibodies to the reovirus haemagglutinin could thus bind to cell surface receptors on uninfected host cells in a manner resembling the virus and could destroy cells bearing complementary receptors. Interestingly, cytotoxic T lymphocytes generated after reovirus infection have been shown to be capable of lysing uninfected B-lymphocyte cells bearing anti-idiotypic-like structures on the cell surface⁹.

The slow virus infections are among the most interesting and least understood of all infectious diseases². This class of diseases, of which scrapie disease of sheep is one example, is characterized by long incubation periods, a progressive degenerative neurological illness and an unusual agent (termed 'prions' by Prusiner and colleagues¹⁰). Partially purified preparations of the scrapie agent contain a 'prion' protein (designated PrP) that is unique to scrapie preparations and may be a structural component of the prion⁵. Insight into the nature of the injury caused by scrapie may come from the observation that prion preparations contain numerous rod-shaped particles that, like amyloid, exhibit a green colour and birefringence by

polarization microscopy. As pointed out by Prusiner and colleagues, amyloid deposits have been seen in a number of degenerative disorders of the central nervous system. A direct link between the putative rods associated with scrapie and amyloid would provide a major clue to the aetiology of a number of degenerative diseases. Unfortunately, the association between prions and amyloid is not yet certain as normal cells contain rod-like structures, and 'prions' have not yet been completely purified⁵.

Clearly, the means by which viruses can injure their hosts are diverse. These include injury due to subtle effects on cell metabolism without overt pathological change, the production of autoantibodies to tissue components or products, and the

deposition of material related to normal cell products. What remains to be determined is which of these strategies of virus-induced injury are important in causing human disease. □

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Natural materials

Chalky submarines

from Paul D. Calvert

MOST of us, if asked to design a submarine, would not think of chalk as a very suitable constructional material. But a recent paper by Birchall and Thomas (*J. Materials Sci.* **18**, 2081; 1983) on the structure of cuttlefish bone illustrates how good design can procure impressive performances from the most unpromising starting materials.

The cuttlefish (*Sepia officinalis*, a relative of the squid) maintains neutral buoyancy at varying depths in the sea by pumping water into or out of the cuttlebone which is divided into small closed compartments. When empty of fluid these cells are at a pressure of about 0.8 atmospheres. Hence they must withstand a high external hydrostatic pressure corresponding to the depth of the fish (1 atm per 10 m).

The cuttlebone structure comprises parallel sheets of calcium carbonate (aragonite) about 10 μm thick separated by 200–600 μm . The sheets are held apart by pillars of the manner of a multistorey car park except that each layer is sealed from those above and below. The whole assembly is supported on a 1.5 mm dense bony dorsal shield.

The obvious design for a pressure-resistant vessel is a thick-walled hollow sphere — just as seen in a bathysphere. A simple calculation for a given compressive strength of the material would give this sphere a density of about 75 per cent of either a layered pillared structure or a cylinder. Birchall and Thomas measure the same crushing strength perpendicular or parallel to the layers as the structure has to resist uniform hydrostatic forces. Apparently, the cuttlefish trades the improved depth resistance that would come from a spherical pressure vessel for an elongated cuttlebone which can also act as an internal skeleton. The pillared layer structure bears a strong resemblance to the

skin-and-honeycomb panels used to obtain maximum rigidity with minimum weight in aircraft and provides a light and rigid skeleton for the cuttlefish.

A problem with using such an intrinsically brittle material as chalk is that it can be quite strong in compression but relatively small tensile loads can produce failure. Much of the design of the medieval cathedrals can be seen as an exercise in minimising or eliminating tensile loads, but the cathedrals did not have to swim around and fight. One way of circumventing the problem is to ensure that local failure does not lead to catastrophic collapse. Crushing tests show that the cuttlebone does fail by a progressive collapse of the layers rather than imploding as would a hollow structure.

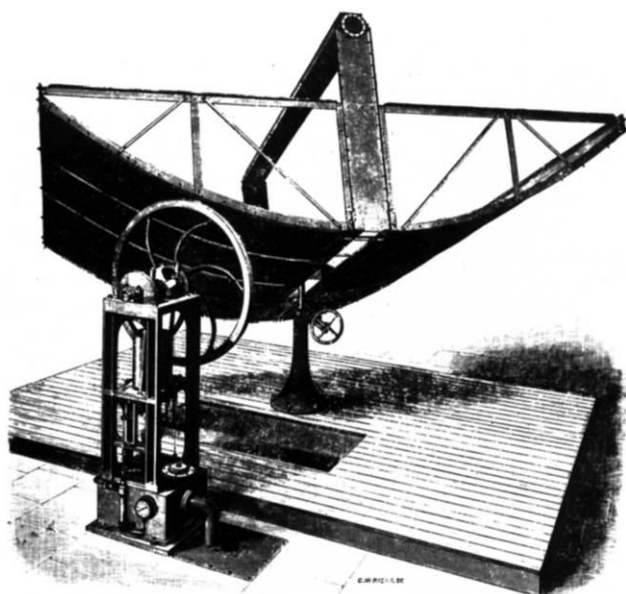
The cuttlefish would also like to get as much tensile strength from the material as possible without sacrificing compressive strength or rigidity. This is also very much a concern in the production of synthetic ceramics both in the context of 'high performance' ceramics, which are the subject of a recent Japanese initiative (*Nature* **305**, 373; 1983), and in the high-strength cements developed by Birchall and his co-workers (*Nature* **289**, 388; 1983). For maximum strength one would like a fine grain size; very small pores, if any; and strong bonds between the grains. The key to the high strength of biological ceramics is their four per cent organic component — a protein-chitin complex which forms a layer covering the carbonate surface. The organic component acts as a nucleating surface for the aragonite crystals and may play a more important part in preventing the crystals from growing too large and/or in improving the bonding between the crystals. S.A. Wainwright and co-authors discussed these ideas well in *Mechanical Design in Organisms* (1976).

The operating parameters for a cuttlefish seem quite respectable when compared with submarines. The cuttlebone crushes at pressures corresponding to a depth of about 230 m and cuttlefish have been recorded at depths of 200 m. The osmotic pump which controls the gas-fluid exchange in the bone would not work beyond about 240 m. By comparison large submarines are reputed to be limited to operating depths of 300 m, which is only about twice their own length, despite being constructed of far stronger materials. It seems almost sacrilegious that such a sophisticated structure as cuttlebone should frequently end up as a budgerigar's beak cleaner! □

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100 years ago THE SUN MOTOR

THE illustration represents a perspective view of a sun motor put in operation last summer. The leading feature of the sun motor is that of concentrating the sun's radiant heat by means of a rectangular trough having a curved bottom lined on the inside with polished plates so arranged that they reflect the sun's rays towards a cylindrical heater placed longitudinally above the trough. This heater contains the acting medium, steam or air, employed to transfer the solar energy to the motor; the transfer being effected by means of cylinders provided with pistons and valves resembling those of motive engines of the ordinary type.



From *Nature* **29**, 217, 3 January 1884.

Observation of stellar remnants from recent supernovae

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The current observational situation regarding the presence or absence of collapsed stellar objects associated with supernova remnants is reviewed. The theoretical expectations and observational evidence for the various possible classes of stellar remnants are discussed. The results of searches for these objects in the radio, optical, X-ray and γ -ray regions of the spectrum are outlined with the conclusion that nine bona fide candidates are currently known. The implications of these results for the origin and evolution of compact objects are summarized.

FIFTY years ago Baade and Zwicky¹ established the distinction between common novae and the vastly more energetic supernovae which they argued must bear visible witness to the destruction of a normal star. They also concluded² "With all reserve, we advance the view that a supernova represents the transition of an ordinary star into a neutron star . . . Such a star may possess a very small radius and an extremely high density". Eight years later, Baade³ and Minkowski⁴ discussed the Crab Nebula as the remnant of the supernova reported by the Chinese in AD 1054, and pinpointed the central star as the compact object powering the Nebula's emission. In Minkowski's words, "Taken altogether, the spectroscopic evidence admits, but does not prove, the conclusion that the south preceding star is the central (exciting) star of the nebula". He went on to place an upper limit on the size of the star of $<0.02 R_{\odot}$.

Theoretical work on the final state of collapsed stellar cores continued over the next three decades, although the observational picture remained static. Then, at the end of the 1960s, the suggestions of Baade and Zwicky were dramatically confirmed with the discovery in rapid succession of radio pulsars⁵ (isolated neutron stars), X-ray pulsars⁶ (accreting-binary neutron stars) and Cygnus X-1 (ref. 7) (presumably an accreting black hole). As the change in gravitational energy for a star between its normal state ($R \sim 10^{11}$ cm) and one of these collapsed configurations ($R \leq 10^6$ cm) is $\sim 10^{53}$ erg, the formation of compact objects must be accompanied by a huge release of energy. The explosion itself is, in at least some cases, visible as an optical supernova, while the small fraction of the energy which couples to the surrounding medium leads to an expanding bubble of shock-heated material, compressed magnetic fields and relativistic particles which we observe at later times as a supernova remnant (SNR).

The modern theory of supernova explosions admits several possibilities regarding the fate of the stellar core. Leventhal and McCall⁸ originally suggested that a remnant white dwarf might be required to explain the late time light curve of Type I supernovae (SN). While this explanation is no longer thought to be relevant, SN explosion models have recently been constructed in which a core of $\sim 1 M_{\odot}$ supported electron degeneracy remains following the ejection of the star's outer layers⁹. The standard models for more massive stars¹⁰ require a central implosion which pushes the electron degenerate pre-supernova core to densities of $\geq 10^{14}$ g cm⁻³ and results in the formation of a neutron star. In very massive stars, the neutron degeneracy pressure of this phase may be insufficient to halt the collapse and a black hole will form. Finally, in some instances such as the carbon detonation¹¹ and pair instability¹² scenarios appropriate for stars in the 4–8 $M_{\odot}$ and 100–300 $M_{\odot}$ mass

ranges, respectively, the core may disrupt completely along with the rest of the star and no stellar remnant is formed. The applicability of these various models to a specific mass range and evolutionary history of SN progenitors is still quite uncertain. Likewise, the properties of the stellar remnants themselves—mass, surface temperature, magnetic field strength and angular momentum—are not well-determined by the theories and we must look to observations of the sites of relatively recent SN explosions to delimit the range of possibilities.

As noted above, a blue star with a peculiar spectrum in the 1,000-yr-old Crab Nebula was the first suggested candidate for the compact remnant of a SN explosion. This suggestion was confirmed 35 yr after it was first made with the discovery that this star was a radio^{13,14} and optical¹⁵ pulsar: a rapidly rotating ($\Omega \sim 200$ rad s⁻¹), magnetized ($B \sim 10^{12}$ G) neutron star. This discovery actually followed by a few weeks the detection of a similar object in the 10⁴-yr-old Vela SNR¹⁶. Despite numerous searches in various wavelength bands throughout the electromagnetic spectrum, however, no further pulsar/SNR associations were discovered between 1969 and 1979, although the circumstantial evidence that a significant fraction of SN must produce neutron stars became compelling. Several hundred more slowly rotating (and presumably older) radio pulsars were discovered, suggesting $\geq 10^5$ such objects existed in the Galaxy and leading to a pulsar birth rate currently estimated as 1 per 40 yr (ref. 17). In addition, 100 of the brightest galactic X-ray sources were found to be binary systems powered by accretion of matter from a normal companion onto an orbiting neutron star or, in perhaps a few cases, a black hole. Neutron stars were also implicated in other discoveries of the decade including 100 MeV γ -ray sources, globular cluster X-ray sources, and γ -ray and X-ray bursters. Integrating a 1 per 40 yr birthrate over the lifetime of the Galaxy, a total galactic population of $\sim 5 \times 10^8$ neutron stars seemed probable.

Comparison of these population statistics with the rate of SN occurrence in the Galaxy (variously estimated as 1 per 20 yr from comparison with the rates observed in external galaxies¹⁸ to 1 per 80 yr from the SNR formation rate¹⁹) suggests that either most SN produce compact stellar remnants or that such collapsed objects can be formed in some less spectacular manner. However, even if all SN do result in the formation of a stellar remnant, there is good reason to doubt that they all result in the formation of a rapidly rotating magnetized neutron star similar to the Crab pulsar. The radio, optical, and X-ray appearance of the Crab Nebula is clearly different from that of most other historical SNR. Its centrally peaked brightness distribution, flat radio spectrum and high degree of linear polarization are understood to result from the injection of relativistic parti-

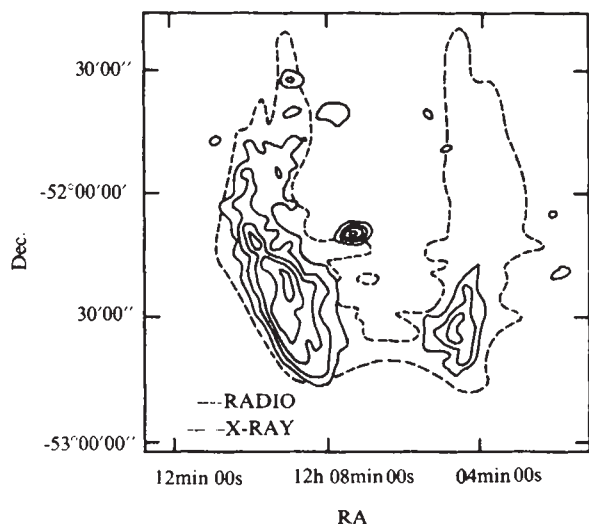


Fig. 1 A radio/X-ray contour plot of the remnant PKS1209-52.

cles accelerated near the neutron star; that is, the presence of the Crab pulsar could have been inferred from the surrounding synchrotron nebula even if the pulsed radiation had gone undetected. Most other historical SNRs show neither a pulsar nor any evidence for pulsar accelerated particles. From this one must conclude that either most SN do not create rotating magnetized neutron stars or that they create such stars in an environment or with characteristics which suppress the formation of a synchrotron nebula.

Examples of this second possibility, that neutron stars exist in SNRs but cannot accelerate particles, include a close binary system with mass transfer, in which the pulsar mechanism is smothered, and a stellar magnetic field outside some allowed range within which the pulsar mechanism operates^{20,21}. We know that neutron stars do occur in binary systems from observations of X-ray pulsars but, until recently, none was known to be associated with a SNR; two have now been found^{22,23}. The idea that some newly-formed neutron stars may suffer from an intrinsic inability to produce high energy particles is a relatively new one and no clear cases have been confirmed, although the possibility that such stars have been detected through their surface thermal emission is not excluded (see below). Within this context, then, we outline below the current status of the continuing search for the stellar remnants of supernovae.

Searches for compact remnants

Radio: The association of radio pulsars with SNRs has rested on the two cases of the Crab and Vela for over a decade, despite the discovery of over 330 other pulsars and the cataloguing of over 140 SNR. The fact that there are no other coincidences is a cause for concern but not, perhaps, alarm. Several observational selection effects work against the discovery of young pulsar/SNR pairs in most pulsar searches: decreased receiver sensitivity resulting from the added noise of the remnant itself, decreased search sensitivity at short periods, the increased severity of pulse smearing for short-period sources due to large dispersion measures and multipath scattering, the large distances to most SNR (50% of the known pulsars are <2.3 kpc away, whereas only ~10% of the SNR are within this distance), and the fact that perhaps as few as 1 in 5 of the pulsars will have beams which intersect our line of sight. High pulsar proper motions are not a problem, as the mean expansion velocity of even a 20,000-yr-old remnant is $\geq 400 \text{ km s}^{-1}$, larger than the largest measured pulsar transverse velocities.

A few targeted searches have been conducted on several nearby SNR. Manchester and Taylor²⁴ catalogue the results through 1976 for 12 remnants ranging in distance from 2 to

10 kpc. The limits are in the range $L_R \sim 5\text{--}10 \times 10^{25} \text{ erg s}^{-1}$, only a few times that of the weakest known pulsar and a factor $>10^3$ less than the radio luminosity of the Crab. More recently, Seiradakis and Graham²⁵ have searched 20 SNR within 8 kpc to a similar sensitivity at 21 cm in an attempt to limit the problems of the SNR background and pulse smearing. However, their lower limit to periods was 300 ms and no new sources were found. Manchester *et al.*²⁶ have just completed a larger 21-cm search in the Southern Hemisphere covering 55 galactic SNR, 30 Large Magellanic Cloud (LMC) remnants, and several other objects, including two Cos B γ -ray sources. At a limiting sensitivity of 1 mJy out to dispersion measures of $1,800 \text{ cm}^{-3} \text{ pc}$, they detected three new galactic pulsars only one of which²⁷ is clearly associated with a SNR—the recently identified X-ray pulsar in MSH15-52.

We need not assume, *a priori*, that all compact sources within SNR will be radio pulsars. In 1973, a variable unresolved, radio source named CL4 was discovered near the centre of the Cygnus Loop²⁸. The source was immediately discussed as a possible stellar remnant, although it was also argued that it was extragalactic²⁹. Very recently, CL4 was identified as a background quasar³⁰. In 1978, a list of nine unresolved radio sources, all located within SNR, were proposed as candidate stellar remnants³¹. The list included two sources previously detected as X-ray objects, SS433 and Cir X-1, as well as CL4. It is now well known that SS433 is associated with the SNR W50 (ref. 22). Cir X-1 is without doubt also a galactic object, but its association with G321.9-03 has never been firmly established. Recent reviews summarizing the remaining sources in the list suggest that the others are all unrelated background objects^{30,32}. Thus, the Crab and Vela pulsars, along with SS433, remained until very recently, the only radio-emitting stellar objects associated with SNR.

Optical: Hind and Plummer³³ were perhaps the first to suggest a specific object as the compact remnant of a supernova explosion: an 11th magnitude variable star near the position of Tycho's SN. Baade³ and Minkowski⁴ were the first to identify such an object correctly when they pointed to the 'south-preceding' star near the centre of the Crab Nebula as the stellar remnant of SN1054. Over the next 40 yr, the search continued for 'peculiar' optical objects in the historical remnants and a variety of candidates have been reported, none of which is now thought to be relevant. The most recent such search reviews many of these early attempts but finds no convincing new candidates³⁴. Following the discovery of optical pulses from the Crab in 1969, several searches of both galactic and extragalactic SNR using fast photometry were conducted. Horowitz *et al.*³⁵ set limits ~ 5 mag fainter than the Crab for any short-period pulsed sources near the centre of Kepler, Tycho, Cas A and 3C58. Papaliolios *et al.*³⁶ conducted a similar search at the sites of 31 recent extragalactic SN without success. More recently, Pennypacker³⁷ has extended the search into the IR (0.9–2.4 μm) to take advantage of lower interstellar extinction; he obtained pulsed IR limits 3–4 mag fainter than the Crab for the remnants studied in ref. 35.

In 1980, an optical search of the remnant of SN1006 resulted in the discovery of a hot blue star ~ 2.5 arc min from its centre³⁸. Subsequent observations identified the object as one of only five known sub-dwarf OB stars³⁹. The offset between the stellar position and the remnant centre would require an extremely high transverse velocity (800 km s^{-1}) for the star, seemingly violating the current proper motion limit for this object³⁸. Recently, very broad symmetric absorption lines have been detected in the UV spectrum of this object, confirming that it is an unrelated background star lying behind the remnant⁴⁰.

There are thus only three optical point sources associated with the remnants of SN: the Crab and Vela pulsars and SS433. Incoherent synchrotron emission in the outer magnetosphere is thought to be responsible for the pulsar light curves⁴¹ and is expected to scale with the neutron star rotation period P as P^{-10} (ref. 42). Thus, sources with $P > 50$ ms will have $m_v > 20$ for distances of 1 kpc, and are unlikely to be detected even if

Table 1 Properties of compact objects associated with SNR

(1)		(2)		(3)	(4)	(5)			(6)	(7)	(8)	(9)							
SNR name		Point source position		Distance (kpc)	L_x (0.2–4 keV) (10^{33} erg s $^{-1}$)	Probability chance superposition			L_R (10^{28} erg s $^{-1}$)	L_{opt} (10^{31} erg s $^{-1}$)	L_γ (10^{34} erg s $^{-1}$)	Ref. for columns							
						(a)	(b)	(c)				2	3	5a	5b	6	7	8	
(a) Galactic	(b) Common	RA (1950)	Dec (1950)			D (r')	IPC rate (counts s $^{-1}$)	$N(>S)$ (per SNR area)											
h min s																			
G39.6–1.8	W50/SS433	19 09 21.3	+04° 53' 53"	5.0	100	65	0.3	$4 \times 10^{-3} c$	6,000	2×10^5	<25	96	97	96	85	98	99	66	
G109.1–1.0	CTB109	22 59 02.8	+58 36 33	4.2	30	24	0.75	$1 \times 10^{-4} c$	<1	<40	<18	46	100	101	23	101	46	66	
G184.6–5.8	Crab	05 31 31.4	+21 58 54	2.0	500 p	5	40	$1 \times 10^{-8} c$	14 p	400 p	20 p	102	103	104	50	105	106	107	
G263.9–3.3	Vela X	08 33 39.3	–45 00 10	0.5	0.3 p	60	0.3	3×10^{-3}	4 p	0.01 p	4 p	108	109	110	57	105	106	107	
G320.4–1.2	MSH15–52	15 09 59	–58 56 57	4.2	50 p	26	0.07	5×10^{-3}	4 p	<1	<18	45	111	111	45	27	45	66	
G130.7+3.1	3C58	02 01 51.1	+64 35 23	2.6	0.5	5	0.01 H	$4 \times 10^{-3} c$	<5	—	<7	52	112	113	52	114	—	66	
G69.0+2.7	CTB80	19 51 02.4	+32 44 52	3	0.2	1	0.02 H	5×10^{-5}	<200	<100	<9	52	91	115	52	115	116	66	
G332.4–0.4	RCW103	16 13 48.3	–50 55 05	3.3	1.0	9	0.02 H	$4 \times 10^{-3} c$	<5 p	<1	<40	94	117	118	119	94	94	66	
G27.4+0.0	KES73	08 37 45	–04 59	26	1,500	4	0.25	$2 \times 10^{-5} c$	<50	—	<700	120	91	120	PC	PC	—	66	
G296.5+9.7	PKS1209–52	12 07 23.5	–52 09 49	1.1	0.2	81	0.08	0.04	<40	<20	<2	TW	91	121	TW	121	93	66	
G6.6–0.2	W28	17 57 55.7	–23 19 59	2.4	0.1	49	0.01 H	0.34	<16	—	<14	122	91	123	122	122	—	66	
G127.1+0.5		01 25 08.3	+62 50 59	3.8	0.2	50	0.01	0.35 c	2,500	180	<14	125	91	124	TW	63	62	66	

p = Limit or detection based on pulsed flux only. Column (2), best available positions for point sources of radiation. Column (3), distances to SNR from reference quoted in column (9); if a range is given, the mean value has been adopted. Column (4), unless quoted directly from the reference cited in columns (9)(5b), the X-ray luminosity in the 0.2–4.0 keV band has been calculated from $L_x = \text{IPC rate (5b)} \times 5 \times 10^{-11} \text{ erg [cm}^2 \text{ counts s}^{-1}]^{-1} \times \exp[D_{\text{abs}}/10] \times 4\pi D^2$ (column (3)), appropriate for sources at distances $D \geq 1$ kpc with a Crab-like spectrum modified by interstellar absorption in a medium of mean density of 1 cm^{-3} . Column (5a), Mean diameters in arc min taken from refs in column (9). b, Einstein imaging proportional counter rates taken from refs in column (9); an *H* following the rate indicates that it was derived from a high resolution imager counting rate and multiplied by a factor of 5, the mean IPC/HRI ratio. c, The number of sources expected *a priori* above the flux in column (5b) within an area the size of the SNR in question, calculated from the expression in the text; a *c* following the expected value indicates that the source is centrally located in the remnant, reducing the probability of chance superposition still further. Column (6), unless quoted directly from the ref. cited in column (9) the radio luminosity has been calculated from $L_R = 4 \times 10^{27} f_{\text{mJy}} \times D_{\text{pc}}^2$ appropriate to a ν^{-1} spectrum integrated over a bandwidth of 3×10^6 Hz. Column (7), unless quoted directly from the ref. cited in column (9) the optical luminosity has been calculated from $L_{\text{opt}} = 2.4 \times 10^{-6} \times 10^{-(2m_v - D_{\text{kpc}}/5)} \times 4\pi D^2$, where m_v is the visual magnitude of the source or the upper limit to the X-ray source counterpart, and an extinction of 1 mag kpc^{-1} has been adopted. Column (8) γ -ray limits calculated using $f[E > 100 \text{ MeV}] = 1 \times 10^{-6} \text{ photons cm}^{-2} \text{ s}^{-1} \times 4\pi D^2 = 10^{34} \times D_{\text{kpc}}^2 \text{ erg s}^{-1}$; in the cases of RCW103 and W28, fluxes of the nearby γ -ray sources CG333+1 and CG006–0, respectively, have been substituted for the Cos B galactic plane survey limit of $\sim 1 \times 10^{-6} \text{ photons cm}^{-2} \text{ s}^{-1}$. Column (9), TW, this work (data published here for the first time). PC, Personal communication from J. Kriss.

their beams are pointed in our direction. If neutron stars are born with very short periods ($P \leq 5$ ms) and this scaling holds, however, they could be visible at distances up to 100 Mpc. A source such as SS433 would be visible in any remnant in the nearer half of the Galaxy. Although it would be instantly recognizable at high spectral resolution, its colours are not unusual and similar objects could have escaped detection.

X ray: The launch of the Einstein Observatory with its complement of two X-ray imaging detectors⁴³ provided a new and powerful tool for the detection of weak cosmic X-ray sources. The high spatial resolution not only increased our sensitivity by several orders of magnitude but also allowed us to discern the presence of point-like sources of emission within extended objects (particularly applicable to SNR). Before 1979, X-ray emission had been detected from the vicinity of all three known stellar remnants within SNR. Images taken with Einstein resulted in the detection of nine additional stellar remnant candidates which are listed in Table 1 along with the original three. Of all these sources, only the objects within the Crab Nebula⁴⁴, G109.1–1.0 (ref. 23), and MSH15–52 (ref. 45), have been observed to pulse in X rays, although the upper limit on pulsations in the other objects is restrictive only for the Vela pulsar. This lack of detected pulses leaves room for some uncertainty in the emission mechanism involved; possibilities include black-body radiation from the stellar remnant's surface, nonthermal emission from the magnetosphere (as is the case for the Crab pulsar), or accretion from a companion star (as is likely for the G109.1–1.0 source)⁴⁶. Unfortunately, most of these sources are quite weak ($\leq 0.01 \mu\text{Jy}$ at 1 keV), and it is unlikely that another opportunity to study them will arise for several years to come.

Despite the success of Einstein in tripling the number of compact sources associated with SNR, it is still true that most remnants contain no point source of electromagnetic radiation. The seven historical remnants (Cas A⁴⁷, Kepler⁴⁸, Tycho⁴⁹, Crab⁵⁰, SN1006 (ref. 51), 3C58 (ref. 52) and RCW 86 (ref. 53)) have been particularly well studied, and only the two Crab-like remnants (the Crab and 3C58) show point source

emission. In addition, the Einstein programme⁵⁴ included 65 other galactic remnants as well as at least 31 confirmed SNR in the Magellanic Clouds⁵⁵. Three of the 25 LMC remnants have centrally-condensed brightness distributions with at least two of the three exhibiting power law X-ray spectra⁵⁶. Limited instrumental resolution does not permit us to resolve any point source components in these objects, but their presence may be inferred by analogy with the galactic Crab-like remnants. None of the other remnants in the Large or Small Clouds shows any signs of an associated point source. In the Galaxy, seven additional Crab-like remnants were observed: point sources were detected in two of them (Vela⁵⁷ and CTB 80 (ref. 52)), two more were detected as X-ray emitters but no point sources were resolved (G21.5–0.9 (ref. 58) and G29.7+0.3 (ref. 59)), and three were too distant to detect at all. Of the 58 shell-type remnants studied (33 of which are within 5 kpc), point sources have been reported within the boundaries of eight. However, these remnants cover a large area of sky and the number of chance coincidences with foreground (stars) or background (active galactic nuclei) interlopers is non-negligible. Table 1 lists the number of sources at or above the observed flux, *S*, to be expected within the boundaries of each remnant calculated from the known surface density *N* of soft X-ray sources⁶⁰.

$$N(>S) = 6.5 \times 10^{-4} \left(\frac{S}{1 \text{ IPC cts s}^{-1}} \right)^{-3/2} \text{ sources deg}^{-2}$$

where a conversion factor of $2.5 \times 10^{-11} \text{ erg cm}^{-2} \text{ s}^{-1}$ per IPC count has been assumed. (IPC is the imaging proportional counter on the Einstein Observatory, see ref. 43.) Three sources have a rather high probability of being chance superpositions: W28, PKS1209–52 and G127.1+0.5. The latter source is already known to be the serendipitous alignment of a radio galaxy at redshift $z = 0.018$ with the remnant's centre^{61,62} (however, see ref. 63 for an alternative view of this object); the sources in W28 and PKS1209–52 (Fig. 1) are significantly offset from the remnant centres. Eliminating the three large nearby remnants which span several square degrees each (Monoceros, Lupus and the Cygnus Loop), we calculate the expected number

Table 2 Sample upper limits on compact objects in SNR

Limits for	Cas A			SN1006			SNR at 5 kpc			Ref. and notes			
Units:	L_{Crab}	L_{Vela}	L_{SS433}	L_{Crab}	L_{Vela}	L_{SS433}	L_{Crab}	L_{Vela}	L_{SS433}	Cas A	SN1006	SNR at 5 kpc	Notes
Radio	0.5 p	1 p	0.5	0.001 p	0.002 p	10 ⁻⁴	0.1 p	0.3 p	0.05	24 p , 127	26 p , 128	*	‡
Optical	0.005	—	10 ⁻⁵	0.001	30	10 ⁻⁶	—	—	—	129	34	—	§
X-ray	0.03	1	0.01	0.002	0.05	0.0005	0.02	2	0.02	47	130	†	
γ ray	0.5	2	—	0.05	0.2	—	1	5	—	66	66	66	¶

p , Limit based on pulsed flux only.

* Assuming (1) a limit on pulsed flux at 400 MHz of 10 mJy, consistent with the sensitivity of recent all-sky pulsar surveys, and (2) a limit of 50 mJy at 1,400 MHz for a radio-point source associated with any SNR and as yet unnoticed.

† Assuming a limit of 0.02 IPC count s⁻¹ for a point source associated with any SNR surveyed with the Einstein Observatory and as yet unnoticed.

‡ For comparison with the Crab and Vela pulsed 400 MHz fluxes, we have used pulsed flux limits from the cited references and computed the distance-corrected ratio for the pulsed luminosity limits (denoted by p); for comparison with SS433 at 1,400 MHz, we have used limits derived from the 1,400 MHz maps cited and computed the distance-corrected ratio for the luminosity limit. Distances of 3.0 kpc and 1.0 kpc were adopted for Cas A and SN1006, respectively.

§ Optical luminosities were calculated as in Table 1 from magnitude limits in the cited references and compared with those quoted in Table 1 (column (7)) for the Crab, Vela and SS433.

|| X-ray luminosities were calculated as in Table 1 from IPC (or HRI $\times 5$) count rate limits in the cited references and compared with those from column (4) in Table 1.

¶ γ -ray luminosities were calculated as in Table 1 from the Cos B galactic plane survey limit in the quoted reference and compared with those from column (8) in Table 1.

of chance coincidences for the sample of 60 shell-type remnants as a whole is ~ 4 , in reasonable agreement with the three probably spurious associations found.

The results of the X-ray survey, then, are that point sources are associated with at least 4 of the 9 Crab-like remnants and at most 5 of the 63 shell-like remnants (5 of 30 within 5 kpc where the survey is complete to a limiting luminosity of $\sim 2 \times 10^{33}$ erg s⁻¹). The completeness of our sample for the various types of point source emitters is discussed below.

γ -ray: The Crab and Vela pulsars were both detected in the first survey of the sky at energies > 50 MeV (refs 64, 65). The most recent catalogue⁶⁶ of localized γ -ray sources from the COS B survey contains 25 entries; the Vela pulsar is the brightest source in the sky in the 0.1–3.0 GeV band and, along with the Crab, is one of only four sources identified with objects known in other spectral regimes. Both sources have also occasionally been reported as detections by ground-based Cerenkov air shower experiments operating at photon energies $> 10^{11}$ eV (ref. 67). Despite earlier reports to the contrary, these are the only two radio pulsars that have been detected above 2 keV, although several workers^{68–70} have suggested that young pulsars form a significant component of the unidentified γ -ray source population.

The whole galactic plane (and, therefore, most galactic SNR) has been covered in the COS B survey to a limiting flux of $\sim 1 \times 10^{-6}$ photons cm⁻² s⁻¹ ($E > 100$ MeV)⁶⁶. For a source with a Crab-like spectrum at a distance of 1 kpc, this corresponds to a γ -ray luminosity of $\sim 10^{34}$ erg s⁻¹ (5% that of the Crab pulsar). We could thus detect any Crab-like source within 4–5 kpc. A semi-empirical prediction of the pulsed γ -ray luminosity from a pulsar magnetosphere is given by $L_{\gamma} (> 100 \text{ MeV}) \sim 2 \times 10^{32} B_{12} P^{-1.7}$, where B_{12} is the pulsar surface magnetic field in units of 10^{12} G and P is the pulsar period⁷¹. Thus, even sources with periods as long as 0.1 s may be detectable out to 5 kpc. Without *a priori* knowledge of the period, however, the counting statistics of the γ -ray maps are too poor to establish the pulsed nature of such sources.

In fact, roughly a quarter of the known localized γ -ray sources are, within their large error circles (diameter ~ 1 – 2°), positionally coincident with catalogued radio SNR^{72,73}, a result of marginal statistical significance. Lamb and Markert⁷⁴ have examined three of the associated remnants at soft X-ray energies with Einstein and find nothing unusual in any of them. Further progress must await new γ -ray observations with improved angular resolution and sensitivity. Observations at these energies offer a unique window on young pulsars as the γ rays may be produced in a fan beam visible to all observers⁷⁵, unlike the narrow pencil beams dominant at lower energies. The very

different pulse profiles of Vela at radio and γ -ray wavelengths and the pulse–interpulse morphology of both the Crab and the Vela at high energies suggest that the γ -radiation arises from farther out in the magnetosphere than does the radio emission and thus is likely to have a substantially wider beamwidth. It has been argued that ‘Gemina’, the brightest unidentified γ -ray source which has a soft X-ray counterpart but no detectable radio emission, is an example of such a γ -ray pulsar whose radio beam is pointed away from us (D.J.H., unpublished data).

We consider the first nine objects listed in Table 1 as highly probable associations of SNR and radiating compact stellar remnants. All are detectable in the soft X-ray band, four are radio sources, three have optical counterparts and two are seen at γ -ray energies. We discuss below the source classes and evolutionary state of these objects; here we summarize the current upper limits to similar sources of radiation in other galactic SNR.

In Table 2 we list, for various spectral regimes, the upper limits on point source emission in several SNR: the youngest (~ 325 yr) known remnant Cas A, the closest (~ 1 kpc) historical remnant SN1006, and a typical remnant at a distance of 5 kpc. The limits are quoted as fractions of the luminosity of the Crab, a 10^3 -yr-old radio pulsar; of Vela, a 10^4 -yr-old radio pulsar, and of SS433, a system which is, to date, observationally unique, but which probably contains an accreting neutron star or black hole.

Perusal of Table 2 leads to a number of interesting conclusions. One can now assert that a pulsar similar to that in the Crab Nebula is not present in any of the nearby SNR. This conclusion holds independent of the radio beaming pattern for two reasons: (1) the theoretical and phenomenological arguments presented above which suggest that pulsed γ rays are radiated in a wide beam visible to most observers; and (2) X-ray synchrotron nebulae similar to the Crab Nebula have now been found surrounding all three young radio pulsars (Crab⁷⁶, Vela⁵⁷, and MSH15–52 (ref. 45)), as well as around several older isolated pulsars^{77,78}. The soft X-ray luminosities of these nebulae are comparable with or greater than the pulsed radiation and would be easily visible within any of the shell-type remnants out to 5 kpc.

A pulsar similar to Vela would also not have escaped detection in any of the well-studied remnants, although a distance of 5 kpc is about the limit to which the various surveys would have detected such an object. Thus, we can conclude that an active radio pulsar born in any of the 36 closest SNR must be radically different from our two canonical young pulsars (for example, $P > 0.1$ s at birth, and a very low magnetic field strength).

Table 3 Supernova scenarios for binary stars

Case	Primary mass ($M_{\odot}$)	Companion mass ($M_{\odot}$)	Resulting system*	Observational consequence
I	>10	>10	(a) SN+>10 (b) SN+>10	Visible: wind-powered binary Visible: wind-powered binary
II	>10	4-10	(a) SN+>10 (b) SN+4-10	Visible: wind-powered binary Visible: wind-powered binary
III	>10	<4	(a) SN+4-10 (b) SN+>4	Visible: wind-powered binary Disrupts
IV	4-10	4-10	(a) SN+>10 (b) SN+4-10	Visible: wind-powered binary Visible: wind-powered binary
V	4-10	<4	(a) SN+4-10 (b) SN+<4	Visible: wind-powered binary Disrupts
VI	<4	<4	(a, b) WD+<4 → SN+evolved star	Visible: Roch-lobe overflow binary

* Case (a) represents conservative evolution wherein most of the material lost by the primary is transferred to the companion whereas in case (b) most of the mass lost by the primary leaves the system. In more realistic treatments of nonconservative mass transfer, the companion star often still accretes sufficient material to reverse the mass ratio of the system and prevent disruption of the binary in the SN event. Thus, low-mass, Roche-overflow driven binary such as Her X-1 can result from a progenitor system with initial masses greater than in case VI (ref. 131).

An SS433-type object with $L \geq 10^{-2}$ of the X-ray or radio luminosity of the prototype is also unlikely to have escaped detection in any SNR out to at least 5 kpc. Likewise, a less spectacular accretion-powered X-ray source such as that in G109.1-1.0 would also be easily visible to that distance in the X-ray band. In fact, for any compact remnant that remains in a binary system, our X-ray survey luminosity limit of $L_X \leq 2 \times 10^{33} \text{ erg s}^{-1}$ allows us to limit the mass loss rate $\dot{M}$ of its companion star as:

$$\dot{M} < 3 \times 10^{-11} M_{\odot} \text{ yr}^{-1} \left(\frac{\zeta}{0.1} \right)^{-1} \left(\frac{M_x}{1.4 M_{\odot}} \right)^{-2} \left(\frac{a}{10 R_0} \right)^2 \times \left(\frac{v_w}{500 \text{ km s}^{-1}} \right)^4 \left(\frac{D}{1 \text{ kpc}} \right)^2$$

where ζ is the fraction of the rest mass energy of accreted matter that is converted to radiation, M_x is the mass of the collapsed object, a is the orbit semimajor axis and v_w is the velocity of the stellar wind⁷⁹. Thus, the main-sequence companions of any such putative close binaries are rather weakly limited by the X-ray survey to those with mass loss rates typical of spectral types later than A5 ($m_v > 7$) for 1 kpc and later than B5 for 5 kpc ($m_v > 12.5$); optical searches for binaries with unseen companions would clearly be a more sensitive method of searching for such objects. Our limits for an isolated black hole or neutron star accreting interstellar material are similarly weak: for spherical accretion⁸⁰,

$$L_X = 7 \times 10^{31} \left(\frac{\zeta}{0.1} \right) \left(\frac{\rho}{1 \text{ cm}^{-3}} \right) \left(\frac{M}{1.4 M_{\odot}} \right) \times \left(\frac{v}{10 \text{ km s}^{-1}} \right)^{-3} \text{ erg s}^{-1}$$

where v is the object's velocity with respect to the infalling matter. Thus, an isolated $10 M_{\odot}$ black hole might be detectable to 5 kpc, but a neutron star (likely to be moving at a considerably higher velocity) would probably not be observed in even the nearest remnants.

Source classes

We have divided the observed compact objects into three groups: accretion-powered binary sources (SS433 and G109.1-1.0), active pulsars as defined by the presence of pulsed radiation and/or a surrounding synchrotron nebula (Crab, Vela X, MSH15-52 3C58, CTB80), and isolated, noninteracting objects (RCW103, G27.4+0.0, PKS1209-51/52, W28, and G127.1+0.5). Within each category, the sources are listed in Table 1 in order of decreasing likelihood that they are true members of their class; amongst the final group, we feel it is rather unlikely that any of the last three objects are actually

associated with the surrounding remnant. Although the survey of galactic SNR in X rays (the preferred band for detection) is incomplete, it is unlikely that any significant new data will be obtained in the near future, so it seems appropriate to consider at this time the statistical implications of the current list of objects.

Binary sources: Although some doubts have been expressed that the nebulae surrounding SS433 and G109.1-0.1 are, in fact, remnants of the SN which created the compact objects we see, the central location and low probability of chance occurrence for these sources is highly suggestive of real association. The sources are radically different from each other in many of their observed properties, but both probably consist of a near main-sequence star and an accreting collapsed companion. While their radio and optical luminosities are very different, both are strong X-ray sources which would have been easily detected (a factor of $>10^2$ above threshold) in any of the 63 shell-type galactic SNR surveyed with the Einstein satellite; SS433 was originally detected by Uhuru and a similar source would have been seen by other all sky surveys out to ≥ 10 kpc, a distance encompassing ~ 100 of the known galactic SNR. Thus, we can conclude that $\leq 3\%$ of SN leave behind systems which, on a time scale less than the lifetime of the expanding remnant ($3-10 \times 10^4$ yr), become accreting X-ray binaries.

As most stars occur in binary systems, these results hold important implications for binary disruption probabilities in the SN event. In Table 3 we summarize the possible pre- and post-SN configurations of a binary system. The stars are divided into three mass regions: $M > 10 M_{\odot}$ (earlier than B3) for which there is general agreement that the star will end its life as a SN; $4 M_{\odot} < M < 10 M_{\odot}$ (B9-B3), a range in which post-main-sequence evolution is particularly uncertain, but within which it is believed that at least some of the stars explode and leave collapsed remnants; and $M < 4 M_{\odot}$ (later than A0), for which there is general agreement that most stars evolve to white dwarfs. Table 3 gives the zero-age main sequence mass range of the primary (more massive) and secondary binary components and lists the post-SN configuration for each of two possible scenarios: first, mass conservation, in which most of the material lost by the primary is transferred to the secondary; and second, extensive mass loss, in which the separation of the stars is sufficiently large that only a small fraction of the primary's mass is accreted by its companion. The final column of Table 3 presents the expected observational consequences of the various scenarios. If the SN does not leave behind a collapsed object (a neutron star or black hole), clearly, no accretion-powered source will be seen. If a collapsed object is produced, we have several possibilities:

(1) The companion is a massive star ($> 4 M_{\odot}$) and, as such, has a stellar wind strong enough to produce an observable X-ray source (cases Ia and b, IIa and b, IIIa, IVa and b, and Va).

(2) The companion has $M < 4M_{\odot}$ (cases IIIb and Vb). However, in this case, as greater than one-half the mass of the system is lost in the explosion, the system will essentially always disrupt.

(3) The SN is produced by accretion onto a white dwarf of matter lost from the companion through Roche-lobe overflow. If the SN leaves a collapsed companion, a visible accretion-powered source will result from the continuing mass loss of the post-main-sequence companion (case VI).

The binary source in G109.1–1.0 almost certainly consists of a neutron star fed by Roche-lobe overflow from a low mass ($< 1M_{\odot}$) companion⁸¹ and thus probably resulted from case VI. We note that this would require a white dwarf SN to leave behind a neutron star remnant, in conflict with some current theories of Type I explosions⁸². The companion of the collapsed object in the SS433 system is uncertain although the weight of the evidence⁸³ seems to favour a low mass companion, again suggesting a case VI scenario.

The lack of visible high-mass accretion sources in SNR suggests that nearly all such systems must disrupt when the first star undergoes a SN. The rarity of binary pulsars ($\sim 1\%$) supports this conclusion. There are ~ 25 massive X-ray binary systems in the Galaxy⁸⁴ which, just after the first SN, should have been embedded in a SNR. That at most one such system is currently seen in this early phase (lasting $\leq 5 \times 10^4$ yr) yields a crude estimate of the lifetime L of massive binaries: $\frac{1}{25} \geq (5 \times 10^4)/L$, implying $L \geq 10^6$ yr, commensurate with the lifetimes of the massive companions powering these wind-driven sources. The rarity of low mass X-ray binaries within SNR shells suggests that disruption in the SN event is similarly likely or, that collapsed objects are rarely formed in such systems (that is, exploding white dwarfs usually leave no remnant).

An interesting similarity between SS433 and G109.1–1.0 is that both show evidence for the ejection of relativistic beams^{85,86}. Of the ~ 100 galactic X-ray binaries not found in SNR, however, only Sco X-1 evidences this phenomenon^{87,88} although most sources have not been studied at comparable sensitivity in either the radio or X-ray regimes. If this lack of detectable beamed emission is not simply an observational selection effect, it suggests that the phenomenon is a relatively short-lived (few per cent) phase of an X-ray binary's evolution. Similar beams are thought to emerge from isolated young neutron stars where they give rise to the synchrotron nebulae discussed below.

Pulsar-driven nebulae: The largest group of detected compact objects associated with SNR are those embedded in centrally peaked radio and X-ray synchrotron nebulae. The Crab Nebula with its central pulsar is the prototype of this class and, in this one case, the total energy budget of the surrounding nebula is successfully accounted for by the rotational kinetic energy loss rate of the neutron star. In other cases, this link has yet to be directly established. Only two of the remaining four objects in this class, Vela and MSH15–52, are radio pulsars for which the spin-down energies have been measured and, in the latter case, the nebula has only been detected at X-ray wavelengths. Another feature which distinguishes the Crab Nebula (and 3C58) from the other members of this class is the lack of a shell of radio and X-ray emission from an expanding SN shock front surrounding the synchrotron nebula. Such shells are present in MSH15–52, Vela and CTB80, as well as in two other remnants in which the presence of a young pulsar may be inferred from the existence of a bright radio and/or X-ray synchrotron nebula: G29.7–0.3 (ref. 59) and G326.3–1.8 (ref. 89). Other cases of pure Crab-like remnants also exist: G21.5–0.9 (ref. 58), and G74.9+1.2 (ref. 90). No systematic observational differences are apparent between the synchrotron nebulae found in shell-type remnants and those in which a shell is absent. Similarly, the shell components of the composite remnants do not appear strikingly different from most SNR in which only the shell is seen. All this suggests that, at least on time scales of $\sim 3 \times 10^2$ – 3×10^4 yr, the two components evolve independently; that is, the presence or absence of an active neutron star (pulsar) has little

effect on the SNR created by the expanding SN blast wave, and the presence or absence of a shell does not affect the formation of a synchrotron nebula.

The implications of such a statement are significant. Consider those remnants which exhibit shells. Only $\sim 10\%$ (6 of ~ 50 well-studied galactic remnants) contain a Crab-like nebula. Because all young radio pulsars studied by Einstein produce such a nebula (Crab⁵⁰, Vela⁵⁷, MSH15–52 (ref. 45), PSR1055–52 (ref. 77) and PSR0355+54 (ref. 78)) we must conclude that no active radio pulsar exists in the other 90% of SNR. Thus, either only $\sim 10\%$ of SN which make shell-type remnants produce neutron stars, or most neutron stars turn on as pulsars after the shell has dissipated^{20,21} ($\sim 30,000$ yr). The former possibility suggests that the convergence of the SNR production rate and the pulsar birthrate is fortuitous, and holds important implications for SN theory. The alternative view of the late pulsar turn-on yields potential information on the evolution of neutron star magnetic fields and the pulsar radiation mechanism. We note that, for a pulsar birthrate of 1 per 40 yr, a significant fraction (0.4–1.0) of SN must leave a neutron star, arguing in favour of this latter alternative.

If we adopt the premise from our observation of composite remnants that only $\sim 10\%$ of neutron stars turn on as radio pulsars within $\sim 10^4$ yr of their formation, we are left to speculate on the inactive counterparts to the shell-less Crab-like remnants—those SN which leave neither shells nor active pulsars. As there are roughly equal numbers of Crab-like remnants with and without shells, the number of inactive neutron stars with and without shells should also be similar; that is, there should be 10 SN which leave no visible remnants for each shell-less Crab-like remnant produced. We are thus led to the rather startling conclusion that nearly half of the SN in the Galaxy leave no promptly observable remnant. We find support for this conclusion in the fact that derived radio remnant formation rates in the Galaxy (1 per 40 (ref. 91) to 80 (ref. 92) yr) have consistently been a factor of ~ 2 below the estimates of the galactic SN rate (1 per 20 to 40 yr)¹⁸. The first recorded extragalactic SN, SN1885 (S Aud) in M31 is noteworthy in this regard; after 100 yr it is still undetectable as a radio source at a limit only a few per cent of Cas A.

Isolated objects: Sources 8 to 12 in Table 1 are the remaining point sources which have been found within SNR boundaries. The evidence for association is simply positional coincidence. We have argued above that sources 10, 11, and 12, located in remnants of large angular size, are most probably chance superpositions of foreground or background objects. The object at the centre of G127.1+0.5 has already been identified as a radio galaxy^{61,62} and work is under way to identify optical and/or radio counterparts to the sources in PKS1209–52 (ref. 93) and W28. For the remaining two sources, the probability of chance superposition is small (Table 1, column (5c)) and decreases even further when their precise central location within the remnants is considered. The remnant G27.4+0.0 is very distant, and little information is yet available on the X-ray properties of the point source at its centre (J. Kriss, personal communication). Either nonthermal pulsed emission from a young pulsar or emission from a synchrotron nebula (as in other composite remnants) are viable possibilities; its X-ray luminosity (although highly uncertain because of the large correction for interstellar absorption in such a distant source) is not dissimilar from the Crab pulsed luminosity. Black-body radiation from a neutron star surface is an unlikely explanation as the required temperature is $\geq 9 \times 10^6$ K, far above that of other neutron stars and the predictions of theoretical cooling calculations. For RCW103, Tuohy *et al.*⁹⁴ have recently argued that surface black-body radiation from a neutron star is the most likely explanation for the X rays observed from the central point source. The lack of an optical counterpart brighter than $m_v = 22$ virtually rules out an accretion-powered source, although owing to the steep period dependence of pulsar optical luminosities, a nonthermal magnetospheric emission mechanism is not excluded. The derived surface temperature under the assumption of black-body

emission is $\sim 2 \times 10^6$ K, consistent with standard neutron star cooling calculations⁹⁵.

Conclusions

A total of nine point sources of radiation have now been established as the compact remnants of recent SN explosions. They represent a variety of the known observational manifestations of neutron stars and black holes: accretion-powered binary sources, isolated radio pulsars with their associated syn-

chrotron nebulae, and surface thermal emission. The statistics of the observed objects along with the equally important stringent upper limits to similar radiation from sources in most known SNR provide important information on the galactic SN rate and the origin and evolution of neutron stars. Future study of these sources will contribute substantially to our knowledge of these exotic endpoints of stellar evolution.

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Cloud structure on the dark side of Venus

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Observations of the dark side of the planet Venus at infrared wavelengths between 1.5 and 2.5 μm have shown it to be anomalously bright in portions of this waveband, and to exhibit structured cloud patterns whose rotation period is longer than that of any other clouds.

WHILE it is known¹⁻³ that the unilluminated hemisphere of the planet Venus radiates at wavelengths near 10 μm with a temperature of 230–240 K, there are no reports of radiation at wavelengths shorter than 3 μm , where thermal radiation should be very weak. We now, however, report observations showing that the unilluminated hemisphere is anomalously bright in this region of the infrared spectrum. We have been able to estimate the rotation period of the features responsible, but cannot offer a definitive explanation of the phenomenon.

We first discovered detectable dark-side radiation at dichotomy (illuminated phase 0.5) in June 1983 using the 3.9 m Anglo-Australian Telescope and IR photometer-spectrometer in its imaging mode. As the planet entered its crescent phase, the structure was easily studied and the observations described here were made on 24 and 25 July (phase 0.23) and 18–23 September (phase 0.19). These observations cover the unilluminated hemisphere near both local sunrise and local sunset, but there are no obvious differences between the cloud patterns in the two cases (Fig. 1). No digital enhancement of the images has been performed so that the contrast is exactly as observed, although our choice of observing wavelength reduced the intensity of the illuminated portion of the disk relative to that of the dark side. At this wavelength, the range of intensity observed on the dark side is 0.03% to 1.0% of the peak intensity.

Observational procedures

The IR photometer-spectrometer is a versatile Cassegrain instrument, and for these observations we used it in its spectroscopy and imaging modes. The InSb detector (0.25 mm diameter) is cooled to 50 K and radiation enters via one of a selection of apertures in the focal plane of the telescope, a filter and a doublet Fabry lens of ZnSe. Samples of the incident data flux are taken over an integration time which may be any integral number of milliseconds from 1 to 10,000.

The filter wheel for the spectroscopic observations contains a circular variable filter with a transmission wavelength which is a function of rotational position. For focal plane apertures less than 3.5 arc s, the bandpass is 0.010 of the wavelength. The spectrum reproduced in Fig. 2 was taken in three portions, corresponding to the three wavebands of atmospheric transmission. In each waveband, the filter was stepped at the end of each integration time, typically 5 seconds being required for a single sweep through the spectrum. The telescope was then driven to a nearby patch of sky (for an account of the d.c. drive electronics, see ref. 4), the spectral sweep repeated and the sky spectrum subtracted from that on the planet. (Since the observations were made at night the sky signal at wavelengths below 2.5 μm was negligible.) The procedure continued for a few minutes in order to derive high signal-to-noise in the faintest portions. The spectra and telluric absorption features were calibrated by observations of a nearby G dwarf star for which accurate photometry is available. The absolute calibration is reliable to 10%, and the detailed shape of the resulting spectrum to significantly greater precision.

When used in imaging mode, the filter remains fixed at a chosen wavelength, the telescope is stepped in a raster pattern in synchrony with the integrations as it moves southwards. Data collection is suspended on its return journey, and irregularities of the telescope's motion at the northern turnaround slightly distort the extreme top of the image, seen here as upward streaking of the northern cusp, an effect enhanced by the high degree of saturation in the illuminated crescent. Successful use of this technique on other planets has been demonstrated⁵.

Each image of Venus required about 3.5 min, and usually six consecutive images were combined to produce the final versions, in which irregularities due to seeing are smoothed out. The features of each final image could always be seen on each of its components. All the images were made in daylight, hence the choice of 2.41 μm to maximize contrast against the sky, but images made on 25 July at 1.7 μm and with a broad bandpass of 2.0–2.4 μm were indistinguishable.

The initial pixel size was 1.0 arc s and the focal plane aperture was 2.0 arc s in diameter. Using UK Science and Engineering Research Council's Starlink software, the images were linearly interpolated to 0.5 arc s pixels, and after coaddition, very light gaussian smoothing was applied. The effective resolution was 2.0–2.5 arc s unless poor seeing interfered.

Spectrum of the radiation

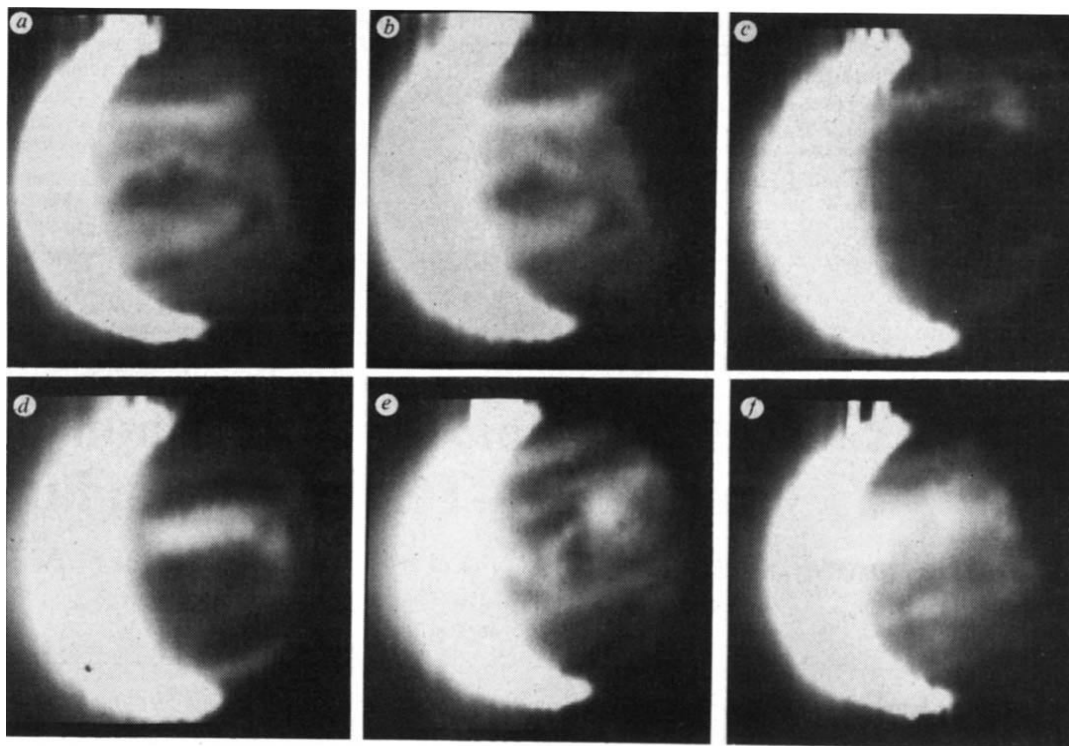
Two issues are raised by this discovery. What is the nature of the radiation seen on the dark side? And what causes the intensity modulation?

Figure 2 shows the spectrum of part of the dark side of Venus between 1.5 and 2.5 μm . The spectrum is contaminated to an extent which cannot be accurately determined by radiation from the sunlit portion scattered along the diffraction spikes of the telescope, but in the portions of Fig. 2 where data are shown as a broken line, we believe the signal arises entirely from this cause because of the exact spectral similarity and the credible value of the intensity. An image made at 2.06 μm , in the absorption band of CO₂, shows no structure on the dark side except a gradual fall-off from the sunlit portion. We therefore believe that the dark side radiation is detectable only in the ranges 1.69 to 1.77 μm and 2.18 to 2.50 μm .

We offer the following explanation of the spectral distribution. In the range 2.3–2.4 μm , the prominent double absorption feature is unmistakably that of the first overtone band of CO, at a mixing ratio of 45 p.p.m. convincingly shown to be present by Connes *et al.*⁶ who required 600 times greater resolution than ours but who observed the sunlit side of Venus. It is clear that we are viewing radiation that has traversed a much greater path length through the atmosphere, in which circumstances we expect bands of CO₂, the most abundant gas, to be highly saturated. In her important study of the atmosphere of Venus, Young⁷ listed all CO₂ bands seen in the data of Connes *et al.* When each one of these is completely opaque, radiation would escape only in the precise wavebands we see, but the peak at 1.74 μm must be further reduced by HCl bands⁸. Apart from very slight changes in the CO band strengths, we find the spectrum of the dark side to be the same in regions differing by a factor of three in intensity.

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Fig. 1 Images of Venus at a wavelength of $2.41\ \mu\text{m}$ made on several dates in September 1983. The bandpass was $0.025\ \mu\text{m}$ and the spatial resolution better than $2.5\ \text{arc s}$. North is at top and east to the left on all images. Dates and UT times are September 1983 at 1720:54 (a), 1723:22 (b), 1921:30 (c), 2101:13 (d), 2120:53 (e), and 2220:16 (f). Several dark spots in image b were caused by electronic faults affecting the raw data.



Using the convenient data of Kuiper⁹ we estimate the column absorption of CO to be equivalent to a 10-m path at atmospheric pressure. When allowance is made for the abundance of CO relative to the other components of the atmosphere of Venus, the observed path must be of the order of 200 km atm. This large value may arise in either a vertical or a dominantly horizontal path. The brightness temperature in the peak of the $1.74\ \mu\text{m}$ region is 450 K, a value found only near the base of the major cloud decks¹⁰, which would be consistent with a vertical path of 200 km atm provided that the absorption optical thickness of the clouds permits the passage of radiation. The clouds are known to have very large scattering optical thicknesses at all optical and infrared wavelengths¹¹⁻¹³.

The only other continuum source available is sunlight, which would have to be scattered from the illuminated hemisphere by the upper cloud layers. Since our images show little indication of a fall in brightness away from the terminator, the scattering could not be accompanied by significant absorption. We note that if such a mechanism were operative at visible wavelengths, the unilluminated side of Venus would be far from dark.

Nature of the clouds

The nature of the cloud structure is far from clear. Superficially, the patterns seen in the IR on the unilluminated hemisphere resemble those seen at UV wavelengths on the sunlit side¹⁴. From observations on successive days during one complete rotation we derive a retrograde rotation period of 5.4 ± 0.1 days based largely on the return of the leading edge of a very extensive dark area seen near the limb. The error does not take account of the considerable changes in the structure of the clouds during one revolution of the planet (compare Fig. 1a and f). Estimates of the rotation period based on the observing period of less than six hours possible each day range from 5 to 7 days. The rotation period is distinct from that of the UV cloud patterns (3.94 days, though with some suggestion of longer periods)¹⁵, and the infrared dipolar feature (3.0 days)¹⁶, but it is similar to that of low-contrast features at 11–15 μm (5.3 days)¹⁷. The pole of the rotation is poorly determined from these data.

The origin of the UV clouds is still not definitely known, but it seems most likely that SO₂ and elemental sulphur, lying in a high-altitude haze, absorb sunlight reflected off the uppermost cloud deck¹⁸⁻²⁰. Moreover, the same cloud deck reflects sunlight between 1 and 4 μm (ref. 21). But since we see no prominent

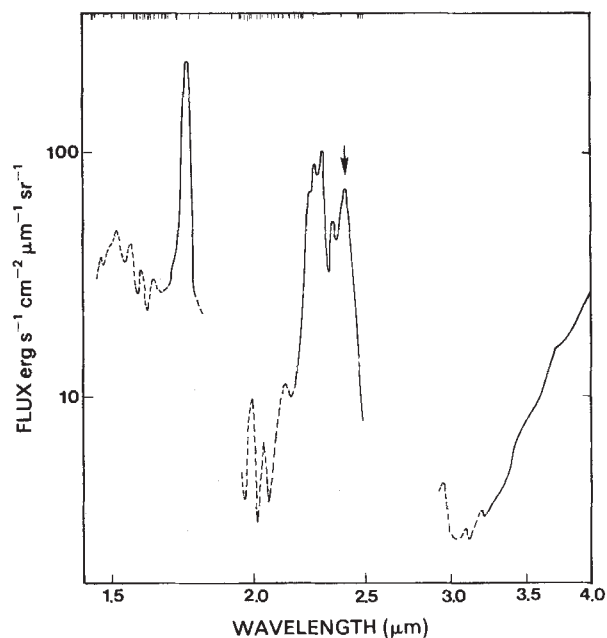


Fig. 2 Spectrum of a small portion of the dark side of Venus, taken on 25 July 1983. Where shown as a broken line the data are dominated by radiation from the sunlit crescent scattered into the beam by the telescopic optics. Beyond $3.3\ \mu\text{m}$ thermal radiation at a brightness temperature of 220 K is seen. Ticks on the upper margin indicate the locations of CO₂ absorption bands. The arrow indicates the observing wavelength for the images in Fig. 1.

cloud structure on the sunlit side at our observing wavelengths, it cannot be the UV absorber that causes the dark-side structure. This conclusion is further strengthened by the fact that neither SO₂ nor elemental sulphur exhibits known absorption in the wavebands of our data except at high overtones.

Markings at very low contrast resembling the UV features, and hence similar to those we now report, have been suspected on the sunlit side at longer IR wavelengths^{22,23}. These are of much lower contrast than the patterns we find on the dark side. Hence we infer that the clouds responsible for the dark-side modulation lie below (or perhaps within) the uppermost cloud

layer. They must therefore have an origin distinct from that of the UV features, but a direct correlation would, nonetheless, be of considerable interest.

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Trace element patterns at a non-marine Cretaceous–Tertiary boundary

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At the fossil-pollen-defined Cretaceous–Tertiary boundary in the Raton Basin of New Mexico and Colorado, an iridium abundance anomaly and excess scandium, titanium, and chromium are associated with a thin ash or dust fallout bed (now kaolinitic clay) that was preserved in freshwater coal swamps.

THE Ir abundance anomaly occurring in a thin zone at the planktonic Cretaceous–Tertiary (K–T) boundary in marine sediments, which was discovered by Alvarez *et al.*^{1,2} and hypothesized by them to be due to fallout from impact of a large extraterrestrial body, has now been reported at numerous sites around the world^{1–11}. Both the evidence and the concept have been challenged, and some opponents have argued that the excess Ir in these sediments can be explained by normal chemical and physical processes in the ocean^{12–17}.

The possibility of another approach to this problem lay in a search for the anomaly in continental sediments deposited in freshwater conditions. In early 1981 we located an Ir anomaly in a core collected in York Canyon, in the central part of the Raton Basin of northeastern New Mexico and southeastern Colorado^{18,19}. The peak Ir concentrations, reaching 5.6×10^{-9} g per g (5.6 ng per g) over a background of 0.004 to 0.020 ng per g, occurred at the base of a thin coal bed within 1 cm of the pollen-defined K–T boundary. Samples taken from above

and below the K–T boundary, including the bases of similar coal beds, showed no evidence of Ir enrichment.

Success in locating an Ir anomaly in the York Canyon core led us to search in outcrops and roadcut exposures along the eastern margin of the Raton Basin. Detailed examination of samples from four of six additional Ir anomaly sites that were located, in addition to the original York Canyon core, has provided us with data regarding the distribution of elemental abundance patterns across the boundary zone, as well as the lithology of the zone. We now report elemental abundance patterns across the K–T boundary over a large area with a common palaeoenvironmental setting in the Raton Basin. We include some supplementary observations made recently on another non-marine K–T boundary, near Jordan, Montana (1,200 km north of the Raton Basin). Interpretation of the data provides clues about the source of the excess Ir and the thin kaolinitic clay bed associated with it.

Measurements

The five Ir anomaly sites for which elemental abundances are reported are shown in Fig. 1 (also shown are two additional sites, Raton (RAT) and Madrid (MAD), at which the Ir anomaly has been found, but for which sampling was not completed). At the outcrop or roadcut locations, after preliminary sampling and palynological examination to establish the K–T boundary, we collected a continuous sequence of samples across the boundary zone at intervals of 0.5–6 cm and at selected levels both above and below the zone (vertebrate fossils have not been reported here); 10–40 cm of surface material were discarded before sampling. Although some of the results for the York Canyon core have been reported previously^{18,19}, these and new data are included because they provide a comparison of elemental distributions observed in an unweathered environment (core) with those observed in the more weathered near-surface conditions at the outcrop and roadcut locations.

All of the abundances except for nickel were determined by instrumental neutron activation analysis. Two portions (3–5 g) from each sample were irradiated with thermal neutrons. One portion was placed in an automated system that provides abundances for about 24 elements²⁰. The second portion was irradiated

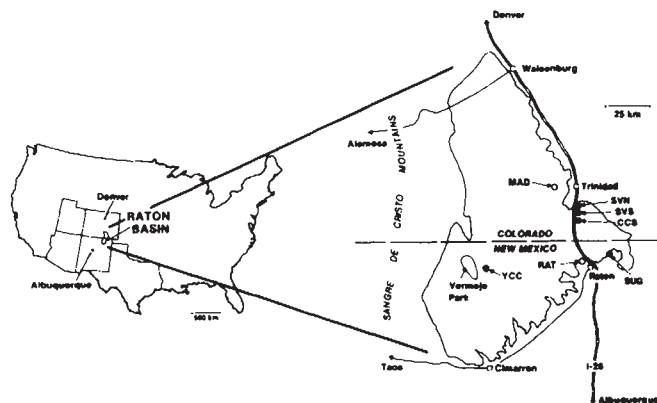
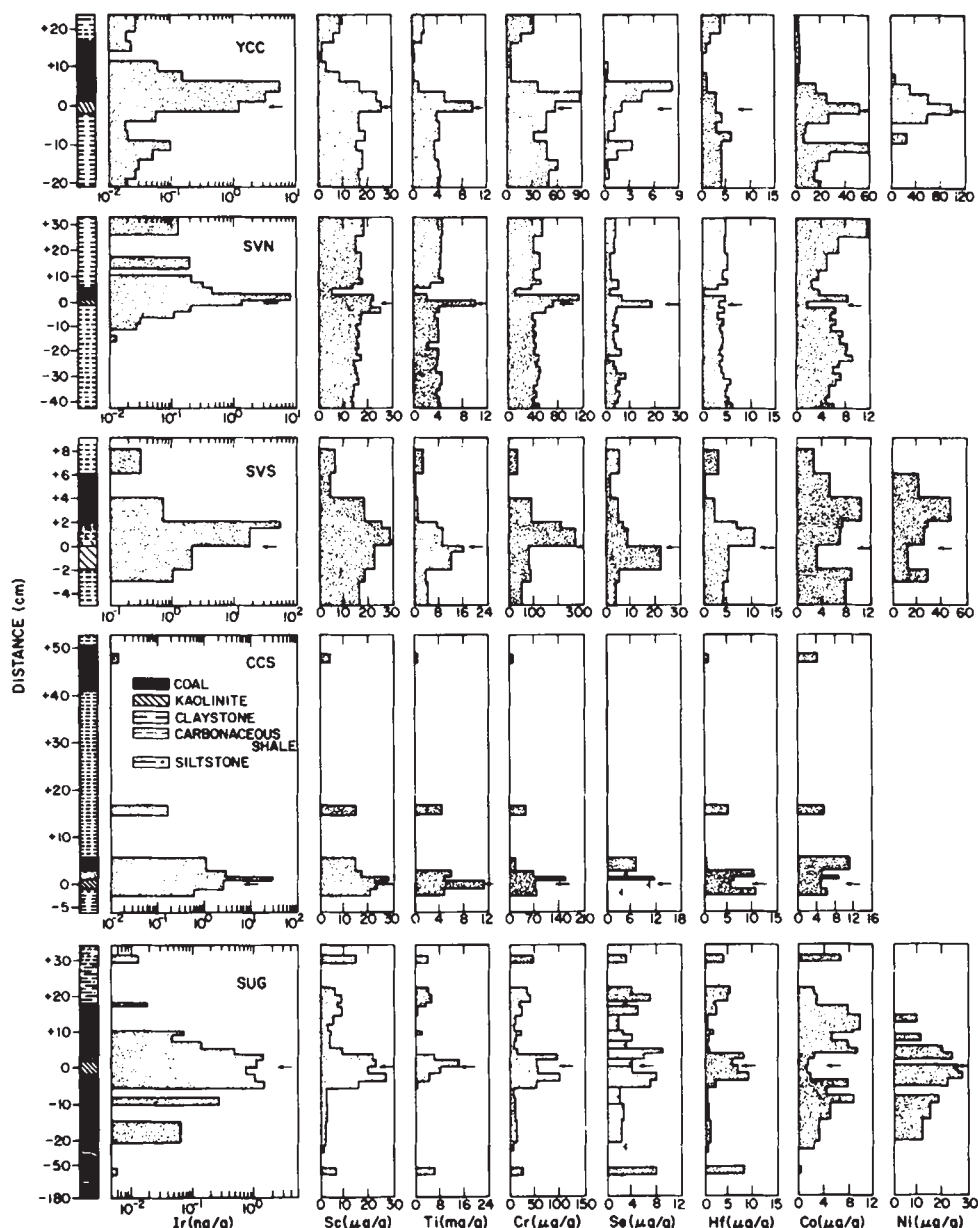


Fig. 1 Outline of present-day Raton Basin and its location in Colorado, New Mexico, and the continental United States. ●, Sites discussed in this work: YCC, York Canyon core; SUG, Sugarite; SVN, Starkville-north; SVS, Starkville-south; CCS, Clear Creek-south. ○, Ir anomaly sites not discussed in this work: MAD, Madrid; RAT, Raton.

Fig. 2 Elemental abundance diagrams for the sites shown with solid circles in Fig. 1. Note that the Ir abundances are plotted on logarithmic scales. The distances up (+) and down (–) section are referenced to the centre of the K–T kaolinitic clay bed (0); arrows indicate this reference level. Vertical bars with left-pointing arrowheads (Se) represent upper limits. Ni was not measured at the SVN and CCS sites.



for 24 h with 1.4×10^{12} neutrons $\text{cm}^{-2} \text{s}^{-1}$ in order to build up the radioactivity level of long-lived species such as 74-day ^{192}Ir . For determination of Au/Ir and Pt/Ir ratios a 1-g portion was irradiated for 7 h with 5.7×10^{12} neutrons $\text{cm}^{-2} \text{s}^{-1}$ and radiochemical separations were performed.

Concentrations of nickel, whose nuclear properties are not well suited for thermal neutron activation techniques, were determined in a few selected samples using a plasma emission spectrometer.

Results

Elemental abundance data for the five sections discussed here are plotted in Fig. 2. At each of these five sites we have found only one Ir anomaly, at the base of the thin coal bed containing the K–T boundary as defined by fossil-pollen changes. This pattern of observations supports the inference that quiet swamp conditions were crucial for the sheltering of Ir-bearing material against dilution by mixing processes. In several closely spaced sections at a site near the city of Raton, the Ir anomaly is rather weak (1 ng per g), and the kaolinite-rich parting, while overlain by a thin coal bed, seems to be contaminated with detrital clay.

In the Raton Basin the palynological K–T boundary interval occurs in a rock sequence deposited in freshwater swamps and flood plains^{18,19,21}. These rocks lie in the lower part of the Raton Formation, of late Cretaceous and Palaeocene age.

At York Canyon, the surface density (total deposition per unit area) of the Ir anomaly shown in Fig. 2 is about 40 ng cm^{-2} . The kaolinite-rich bed at the base of the coal is not visibly distinct from the underlying mudstone, owing to darkening from carbonaceous material; the presence of the kaolinitic clay was determined by X-ray analysis and, once its elemental abundance pattern was established from the elemental systematics of the other K–T kaolinite-rich beds in the basin, by that pattern.

At the Starkville–North Section (SVN), in a roadcut south of Trinidad, Colorado, the kaolinitic bed at the K–T boundary is clearly recognizable, because it has weathered to a grey colour and is located between coal and dark brown carbonaceous shale. The Ir surface density is about 50 ng cm^{-2} , and the Ir concentration reaches a maximum of 8 ng per g.

The Starkville–South Section (SVS) was taken from a roadcut about 500 m south of SVN. Here the Ir concentration reaches 56 ng per g, the highest we have observed in the basin, in a thin (5 mm) shaley bed between the base of the coal and the kaolinitic-clay layer. The Ir surface density here is $\sim 120 \text{ ng cm}^{-2}$.

The Clear Creek–South Section (CCS) was taken about 2 km south of SVS. The Ir concentration shown in Fig. 2 reaches a maximum level of 28 ng per g in a thin (2 mm) carbonaceous-kaolinitic shale that lies on the kaolinitic clay stone bed, and the surface density is $\sim 50 \text{ ng cm}^{-2}$.

Table 1 Elemental abundances associated with the Sugarite kaolinitic clay bed compared with various meteorites and terrestrial and lunar rocks

Element*	Sugarite†	Crustal average (ref. 35)	Lunar soil (refs 36–44)	Lunar basalt	Achondrite eucrite average (ref. 45)	Chondrite CI average (ref. 46)
Na (%)	0.3	2.8	0.30–0.37	0.26–0.36	0.35	0.48
Mg (%)	0.3	2.1	3.8–7.2	3.5–4.9	4.3	9.6
Al (%)	17	8.1	6–9	3.8–14.7	6.5	0.86
K (%)	0.35	2.6	0.11–0.14	0.04–0.22	0.03	0.06
Ca (%)	0.4	3.6	8–9.7	7.1–9.3	7.3	0.9
Sc	34	22	54–92	72–96	26	5.8
Ti (%)	1.35	0.44	4.3–5.6	6.0–7.5	0.46	0.044
V	175	135	34–58	63–92	75	57
Cr	100	100	1,850–1,950	2,300	2,300	2,650
Mn	44	950	1,340–1,820	3,900	3,900	1,960
Fe (%)	0.8	5.0	12	14.4	14.4	18.5
Co	7	25	12–37	2	2	510
Ni	50	75	170–200	20	20	11,000
Se	4	0.05	0.4	0.25	0.25	18
Rb	≤15	90	3	5.7	0.3	2.3
Sr	60	375	165	146–210	75	7.9
Cs	0.8	3	0.1	0.2	0.01	0.02
Ba	540	425	150–430	140–450	30	2.2
La	44	30	11–22	16–43	3	0.24
Ce	74	60	46–68	34–84	—	0.62
Yb	2.4	3.4	12–22	11–18	1.8	0.16
Hf	8	3	8–15	11–20	0.4	0.12
Ta	1.3	2	0.9–2.1	1.6–2.7	0.008	0.02
Ir (ng per g)	3.2	≤1	5–11	0.04–0.50	2–20	480
Th	17	7.2	1.4–2.6	0.6–3.7	0.2	0.03
La/Yb	18	8.8	0.9–1.0	1.4–2.3	1.7	1.5

* Concentrations in $\mu\text{g per g}$ unless noted otherwise.

† See text for discussion of this analysis.

The section from the Sugarite site (SUG), located 10 km east of Raton, includes a 2-m-thick coal bed. The Ir surface density is about 32 ng cm^{-2} . This section is of particular interest because the Ir-enriched zone is sandwiched between two layers of coal and appears to be relatively uncontaminated by detrital clay. Figure 2 shows that the Ir concentration reaches its maximum (1.5 ng per g) not in the thin kaolinitic layer, but in the adjacent coal just above and below. A similar displacement is seen at the other four sampling sites: the Ir concentration reaches its maximum in the coal slightly above the clay layer. This distribution is consistent with spreading due to a combination of at least two dispersion mechanisms: (1) mixing with plant matter following deposition of Ir-bearing fallout; and (2) chemical migration in the acid environment associated with the transformation of plant matter to coal. Iridium concentrations in 27 other non-K-T coal beds and 21 other kaolinitic beds examined in the Raton Basin are $<0.040 \text{ ng per g}$.

The abundance for each element in the SUG K-T kaolinitic clay bed alone was estimated by integrating its net surface density (coal bed background subtracted from the total measured) over individual sample widths and dividing by the surface density of the clay layer (8.8 g cm^{-2}). These abundances are compared in Table 1 with crustal averages and with lunar soil and basalt, chondrites and achondrites. Figure 2 indicates that at the SUG site boundary, Sc, Ti, Cr, Hf and Ni are enhanced in this layer relative to their values in the strata above and below.

Of the elemental abundances measured, four show strong positive correlation with Ir: Sc, Ti, Cr and Pt. Analysis of all data taken in the K-T kaolinitic clay zone shows the following correlation coefficients: $\text{Sc/Ir} = 0.93$, $\text{Ti/Ir} = 0.95$, $\text{Cr/Ir} = 0.96$, $\text{Pt/Ir} = 0.98$. Two other siderophile elements measured, Co and Ni, exhibit no significant correlation with Ir at any of the outcrop sampling sites. Only in the York Canyon core can a clear positive correlation with Ir be observed for these two elements. All of the anomaly sites in the Raton Basin apparently had a common genesis, and thus we suspect that the lack of correlation for Co and Ni with Ir in the outcrop and roadcut sites is associated with their exposure to oxidizing and leaching conditions.

We have measured Pt and Au in eight samples from the York Canyon core and also some from three of the roadcut sites. In

all samples measured the Pt correlated very closely with the Ir, but the Au did not. The concentration of Au peaks near the Ir spike but does not drop to very low levels on either side. Instead the Au background concentration remains at several ng per g throughout the sampled sections.

In Table 2 Pt/Ir and Au/Ir data for the peak Ir samples at four Raton Basin sites are compared with corresponding data for the Stevns Klint, Denmark, and Caravaca, Spain, K-T boundary clay, terrestrial volcanic rocks, lunar soil, and CI chondrites. Unfortunately, there is a paucity of Pt/Ir data for terrestrial basalts, due probably to the difficulty of measuring Pt at very low concentrations. The Au/Ir ratios for the Raton Basin are higher than observed in the marine K-T sites; however, they are distinctly lower than the Au/Ir ratios reported for terrestrial basalts. Finally, we see that the Pt/Ir ratios at our measured Ir spikes fall around the value for CI chondrites.

In every Raton Basin sampling site where an Ir anomaly has been found, the anomaly and the pollen break are associated with a 2- to 4-cm kaolinite-rich clay layer overlain by coal. We have performed elemental analyses on this K-T kaolinite-rich clay bed at six sites, and on 21 other thin kaolinitic clay layers found in other coal beds from about 50 m below to about 300 m above the one containing the K-T boundary. The data are summarized in Table 3. The eight trace elements from Sc to Sb, as well as Ir, are higher in the K-T boundary kaolinitic clay bed than in the others. Although thin kaolinitic clay layers of this kind (commonly referred to as tonsteins) are identified as the alteration product of volcanic ash falls^{22,23}, the distinctively different trace-element character of the K-T layer suggests a different origin.

A similar pattern emerges from results obtained at another group of freshwater K-T boundary exposures, 1,200 km to the north, near Jordan, Montana. From this area, in which the K-T Ir anomaly was first reported by Alvarez²⁴, we have examined samples collected by Bohor and Triplehorn from Brownie Butte, in the vicinity of Hell Creek, where Tschudy²⁵ established the palynological K-T boundary at the lowest lignite bed of the Tullock Member of the Fort Union Formation. Here the Ir anomaly is associated with a 0.5- to 1.5-cm kaolinitic clay bed overlain by several centimetres of lignite. Although the Brownie

Table 2 Atom-ratio data for Ir, Pt and Au

Location	Ratio	
	Pt/Ir	Au/Ir
YCC	3 ± 1	1.1 ± 0.2
SVN	1.7 ± 0.4	0.39 ± 0.06
SVS	2.4 ± 0.5	0.64 ± 0.10
CCS	1.6 ± 0.4	0.64 ± 0.11
Stevens Klint, Denmark (ref. 3)	0.41 ± 0.07	0.20 ± 0.02
Stevens Klint, Denmark (ref. 5)	1.0–1.5*	0.12–0.20*
Stevens Klint, beds 3 and 4 (ref. 10)	2.01 ± 0.07	0.281 ± 0.007
Caravaca, Spain (ref. 10)	1.79 ± 0.16	0.303 ± 0.022
Ultramafic nodule, Globe, Arizona	1.6 ± 0.4	0.17 ± 0.03
Terrestrial basalts (refs 47, 48)	—	15–100*
Lunar soil fines (ref. 40)	—	0.23–0.6*
CI chondrite (ref. 46)	1.75	0.30

* Values shown indicate ranges.

Butte location was in a different floral province during the Cretaceous, the fossil pollen taxon *Proteacidites* occurred in this area and in the Raton Basin. At both locations we have found that this late Cretaceous marker pollen disappears at the top of the clay layer containing the Ir anomaly.

The higher abundance of Ti in the K–T kaolinite-rich clay bed is of some interest. Apart from considerations of its origin, this distinctively higher concentration provides a useful marker for preliminary identification of the boundary bed. In the neutron activation analysis procedure, Ti abundances are determined within minutes, whereas the Ir measurements require long-term irradiation followed by lengthy radiochemical analysis, and thus a high Ti value offers an early alert.

In previous discussions of the Ir enrichment phenomenon, consideration has been given to the possibility that the anomalous material at the K–T boundary may have arisen from volcanism^{13,16,17}. The period from the late Cretaceous through the early Tertiary was marked by a high level of volcanic activity in the western interior of North America²⁶, which could have led to a kaolinitic clay layer of the kind found at the Raton Basin K–T boundary. We have attempted to gain some information on the plausibility of this source by comparing the elemental abundances of the K–T kaolinitic clay bed with those of similar beds above and below it (Table 3), and with those of various other volcanic rocks (Table 4). Table 3 shows that Sc, Ti, Cr and Ir are distinctly more abundant in the K–T bed. The light to heavy rare earth ratios, La/Yb, on the other hand, are about the same and in a range characteristic of crustal rock. The

Table 3 Elemental abundances in thin kaolinitic clay beds in the Raton Basin*

Element†	K–T boundary bed		Beds above/below K–T boundary	
	Range	Average	Range	Average
Al ₂ O ₃ (%)	24–36	32.9	23–36	31.7
K (%)	0.2–1.1	0.52	0.2–2.5	0.81
Sc	21–26	23.3	3–12	6.0
TiO ₂ (%)	1.38–2.67	2.00	0.40–0.95	0.757
V	110–187	137	10–67	27
Cr	46–102	67.3	0.9–5.0	3.3
Co	1.2–53	9.8	0.7–4	2.7
As	1–95	36	0.2–46	4.1
Se	2–19	8	<0.1–6	4.8
Sb	0.3–11.5	6.3	0.1–0.8	0.38
La	16–80	43	9–88	28
Yb	0.7–2.2	1.5	0.8–4.1	2.1
Hf	3.3–6.4	4.5	3.2–10.6	7.6
Ir (ng per g)	0.90–2.7	1.7	0.005–0.020	0.010
Th	5–21	7.8	5–34	9.2
La/Yb	15–57	28.7	5–67	13.3
TiO ₂ /Al ₂ O ₃	0.056–0.074	0.060	0.016–0.027	0.024
Cr/Al ₂ O ₃ ‡	1.64–2.83	2.04	0.036–0.147	0.103

* Boundary data from six sampling sites, non-boundary data from 21 sites.

† Concentrations in µg per g unless noted otherwise.

‡ Cr/Al₂O₃ relative (p.p.m./%)

enhanced concentrations of Sc, Ti and Cr in the Raton boundary clay suggest that this bed may represent the alteration product of basalt or the ash from a basic volcano. Spears and Kanaris-Sotiriou²³ have reported that in British and other European Carboniferous-age tonsteins, the ratios TiO₂/Al₂O₃ and Cr/Al₂O₃ remain virtually unchanged as the volcanic ash alters, and basic ash gives high values for these ratios whereas acid ash gives low values. They point out that basic ratios can be lowered by the addition of sedimentary material. On the basis of TiO₂/Al₂O₃ ratios in Table 3, the K–T kaolinitic layer would have to be ascribed to a basic volcano, whereas all the other beds would have to be ascribed to acid volcanoes.

Of the mafic volcanic and plutonic rocks listed in Table 4, only the dunite and the ultramafic nodule contain Ir in concentrations comparable with those found in the K–T clay layer. However, their Cr concentrations are an order of magnitude too high. Thus, we have difficulty constructing a volcanic source

Table 4 Elemental abundances for K–T boundary clay and for various igneous rocks

Element*	K–T clay Raton Basin†	K–T clay Gubbio, Italy‡	BCR-1 Columbia River basalt§	BHVO-1 Kilauea basalt¶	BIR-1 Iceland basalt	East Pacific Rise #	DTS-1 dunite§	Ultramafic nodule Globe, Arizona**	Raton diatreme***††	Raton Basin sill**	AGV-1 andesite§‡‡
Al ₂ O ₃ (%)	24–35	17.3	13.3	13.9	14.8	15.6	0.32	1.87	6.42	14.9	17.1
TiO ₂ (%)	1.4–2.7	0.78	2.12	2.65	0.93	1.32	0.013	0.28	1.38	2.13	1.05
Sc	21–26	10.0	33	30	45	38	3.5	9.6	27	11.6	12
Cr	46–102	97	17.4	315	350	485	3,920	2,400	1,500	130	12
Co	1.2–53	46	38	46	48	—	139	106	65	19	15
La	16–80	32.4	28	17.5	0.84	2.76	0.029	4.0	110	31.2	38
Yb	0.7–2.2	2.4	3.53	1.6	1.8	2.72	0.015	≤0.4	≤2.5	0.8	1.67
Hf	3–6	2.5	5.0	4	0.66	2.3	0.01	0.70	4	2.8	5.1
Ir (parts per 10 ⁹)	3.2–15	9	0.0035	0.055	0.067	—	0.83	3.0	0.20	0.032	0.011
Th	5–21	7.8	6	0.85	0.60	0.3	0.01	0.7	22	2.5	6.5
TiO ₂ /Al ₂ O ₃	0.056–0.074	0.045	0.159	0.190	0.063	0.084	0.040	0.151	0.215	0.143	0.062
Cr/Al ₂ O ₃	1.6–2.8	5.6	1.31	23	24	31	12,250	1,283	234	8.7	0.70
Sc/Al ₂ O ₃	0.75–0.80	0.58	2.5	2.2	3.0	2.4	11	5.1	4.2	0.78	0.70
La/Yb	15–57	13.5	7.9	11	0.47	1.01	1.9	≥10	≥44	39	22.8

* Concentrations in µg per g unless noted otherwise. Cr/Al₂O₃ and Sc/Al₂O₃ relative (p.p.m./%).

† This work, the Ir abundance represents the Ir surface density divided by the thickness and density of the K–T clay.

‡ This work, sample provided by A. Montanari and W. Alvarez.

§ US Geological Survey standard rock.

|| Data from ref. 49, except Ir from this work.

¶ Data from ref. 50, except Ir from this work.

Data from ref. 51.

** This work.

†† This diatreme, located 2 km east of Raton, was discovered by G. T. Scott (USGS Denver) who provided samples.

‡‡ Data from ref. 49.

that yields the elemental abundance patterns we observe in the Raton Basin K-T boundary bed. Also, basic volcanoes are usually less explosive than their acidic counterparts.

The enrichment of Sc, Ti, Cr and Pt-group elements in the K-T kaolinite also suggests a similarity with lunar surface material. Ruderman and Truran²⁷ have proposed that the K-T fallout resulted from capture by the Earth of lunar surface material ejected by intense photon heating from a nearby supernova burst. The plausibility of this scenario, however, is contradicted by the rare earth measurements shown in Tables 1 and 3. The observed light/heavy rare earth ratios (La/Yb) for our boundary zone samples lie in the range 8–57, characteristic of the Earth's crust. Reported lunar rocks range from 0.9 to about 3, clearly distinct from the former.

Conclusions

If we accept the postulate that the excess Ir is derived from impact of a 10-km diameter asteroid with Type 1 chondritic composition² our Ir data can be used to estimate the fraction of the kaolinitic bed contributed by the asteroid itself. The total integrated Ir under the anomaly peak, expressed as a surface density, was divided by the measured surface density of the kaolinitic clay layer to give the concentration of the Ir in the layer (with the assumption that all the Ir came in with this layer), and comparison of this concentration with that for a Type I chondrite yielded the asteroid contribution. This contribution ranged from 0.6 to 2.5% for the five Raton Basin sampling sites. The corresponding contributions for the Gubbio and Stevns Klint K-T anomaly clays have been reported²⁸ to be 1.6% and 21%, respectively. If we exclude major contributions from nearby volcanic eruptions and aeolian material, the remainder ($\geq 97.5\%$) of the Raton Basin K-T clay layer can be assigned to ejecta from the impact. The elemental abundances of the lithophiles Sc, Ti and Cr, therefore, reflect the composition of the impact site. Our data are best fitted by ejecta from basalts. The La/Yb ratio is high for oceanic basalt, but could have been made up by contribution from overlying sediments.

From our elemental abundance data we are unable to conclude whether or not the palynological K-T boundary is synchronous with the marine boundary, but there exist palaeomagnetism measurements bearing on this question. The palaeomagnetic polarity sequence for marine rocks has now been established for the late Cretaceous through the early Tertiary^{29,30}, and during the boundary period the polarity was reversed. However, Payne *et al.*³¹, measuring samples taken from the K-T boundary zone in the Raton Basin, have reported normal polarity. More recent measurements on samples from the same sites and others in the Raton Basin by Shoemaker *et al.*³² lead to assignment of reversed polarity. The latter work emphasizes that the polarity assignment is critically dependent

on the removal of the strong normal overprint from the palaeomagnetic signals observed in near-boundary-level rocks in the Raton Basin.

The recent investigations at the K-T boundary in Montana have provided some clues to the synchronism of the two continental K-T boundary sequences. We have already pointed out that the elemental abundance patterns for Raton and Montana are similar and that the Cretaceous pollen *Proteacidites* disappears precisely at the top of the kaolinitic boundary clay. Also, Archibald *et al.*³³ have found that the Montana boundary zone lies in a stratigraphical sequence with reversed palaeomagnetic polarity. The highest dinosaur fossil horizon reported³⁴ in this region of Montana occurs ~3 m below the palynological K-T boundary and Ir anomaly, but a statistical analysis of the '3-m gap' data shows²⁴ that one cannot conclude that the dinosaurs disappeared before the Ir layer was deposited.

The body of information we have derived from the elemental abundances, clay mineralogy, stratigraphy and pollen data lead us to the following conclusions:

- (1) The Ir-bearing kaolinite-rich clay bed at all the sampling sites resulted from fallout from a common source, and the excess Ir is associated primarily with the fallout and not with chemical concentration of pre-existing amounts in the local medium.
- (2) The excess Sc, Ti, V, Cr and possibly Hf appear also to have come with the fallout and not from local enrichment processes. This enrichment pattern is consistent with the patterns observed in basalts.
- (3) Although the kaolinite precursor material was deposited on several different local surfaces, in each case it is now overlain by a thin coal bed. This mode of deposition may simply be an indication that quiet swamp conditions preceding coal formation were a favoured environment for the preservation of this fallout material.
- (4) The narrowness of the Ir distribution and the relatively small variation in surface density (a factor of four) indicate that the Ir has not undergone any large scale migration, but may have spread locally from the clay layer into the adjacent material.
- (5) The uniformity in thickness of the K-T kaolinitic clay bed over a distance of at least 1,200 km indicates that the fallout material came from a common source at a considerable distance.
- (6) Although the boundary clay bed may contain some altered volcanic ash or aeolian material, such contribution to the excess Ir does not appear to be significant.

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Coupled Sm–Nd and U–Pb systematics in late Caledonian granites and the basement under northern Britain

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The British late Caledonian granites display a close correspondence between inherited U–Pb systematics in zircons and initial ϵ_{Nd} indicating that both were controlled by the age of a major component of continental crust. The data confirm and refine previous models of a basement comprising young crust in the south which includes arc and oceanic crust scrapings and progressively older continental crust northwestwards across Scotland.

MUCH recent research effort and speculation has been concentrated on the origin of granites, and Nd isotope geochemistry has played a key role in this research^{1–5}. The Caledonian granites of the British Isles are of particular interest in this respect for several reasons. First, they are classical and as internationally recognized examples it is important that their genesis is properly understood. Second, there is a good background of reconnaissance chemical and isotopic data. Third, the Caledonian granites are not easily related to active subduction and there is no obvious mechanism for their generation. Finally they are by far the largest samples of what the lower crust was like at 400 Myr and their isotope geochemistry can tell us a great deal about the regional deep geology of the British Isles.

Pidgeon and Aftalion⁶ discovered a marked Pb isotopic memory in zircons extracted from late Caledonian granites north of the Highland Boundary Fault (Fig. 1) which is absent from zircons from granites to the south. This has been interpreted as reflecting a contrast in basement to the north and south⁶. Pb, Sr, O and Nd isotopic data has confirmed the importance of

continental crust in the generation of these granites^{8–10}. In particular, O and Sr isotopic compositions plot with a broad correlation between the fields for mantle and crust reservoir types^{8,10,11} suggesting overall interaction between mantle-like and crust-like reservoirs during magmatism. The common Pb isotopic compositions generally become more radiogenic southwards implying the southerly diminution of old granulite facies material in the crust^{9,12}.

Despite the large amount of isotopic data available there has been considerable controversy over the origin of the late Caledonian granites. Some authors have argued that the granites were largely derived from mantle, especially those lacking inherited zircons and with low initial $^{87}\text{Sr}/^{86}\text{Sr}$ (refs 13–16), others have proposed crustal source regions^{4,6,7,17} and still other mixed origins but involving mantle or mafic lower crust^{8,10,11,18}. Some of these views have been discussed elsewhere¹⁹.

New Nd isotopic data are now presented which expand the previous data base⁴. From this it is concluded that although a mantle component is important, the lower and middle continen-

Fig. 1 Map of Scotland and northern England showing distribution of Caledonian granites. The name of the granite is followed by the ϵ_{Nd} value(s) and (in brackets) the number of samples.

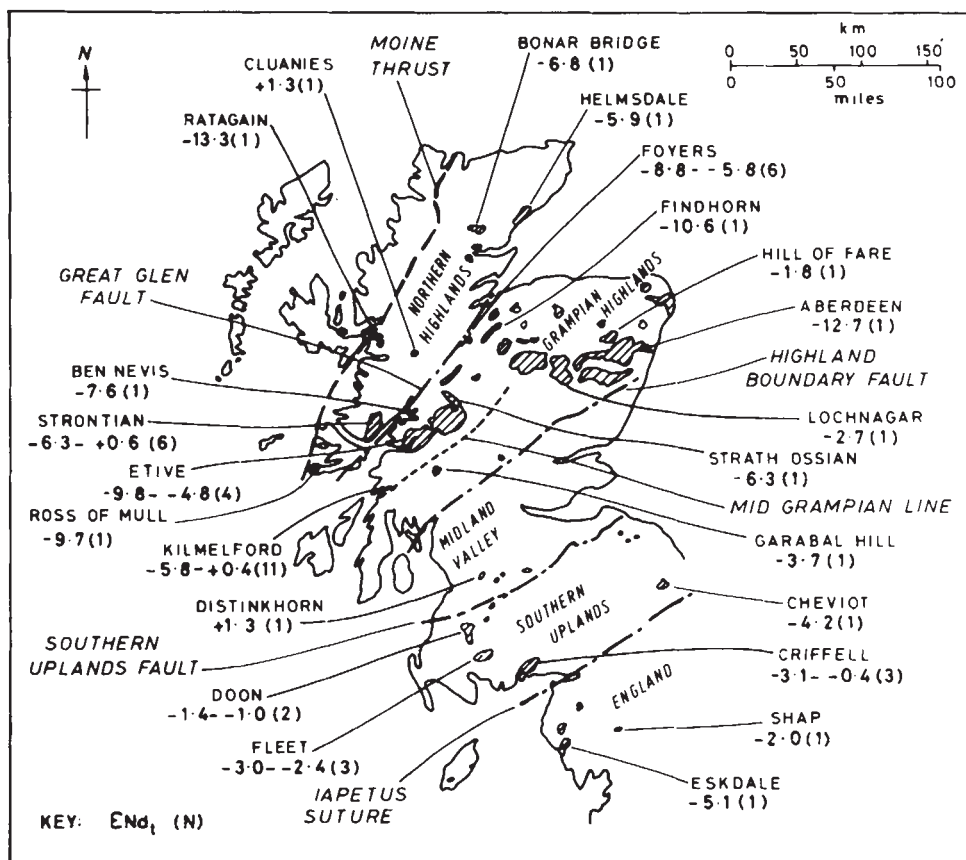


Table 1 Sm–Nd and Rb–Sr isotopic data

Sample no.	Locality	Lithology	<i>t</i> (Myr)	Sm (p.p.m.)	Nd (p.p.m.)	$\left(\frac{^{147}\text{Sm}}{^{144}\text{Nd}}\right)$	$^{143}\text{Nd}/^{144}\text{Nd} \pm 2\sigma_m$	$\left(\frac{^{143}\text{Nd}}{^{144}\text{Nd}}\right)_i$	ϵ_{Nd_i}	$\left(\frac{^{87}\text{Rb}}{^{86}\text{Sr}}\right)$	$^{87}\text{Sr}/^{86}\text{Sr} \pm 2\sigma_m$	$\left(\frac{^{87}\text{Sr}}{^{86}\text{Sr}}\right)_i$	ϵ_{Sr_i}
RC 973	Helmsdale	Granite	420	4.804	33.77	0.08597	0.512028 ± 19	0.51179	−5.9	0.8309	0.71118 ± 5	0.70621	+31
RC 720	Ratagain	Monzonite	425	5.119	31.62	0.09784	0.511681 ± 32	0.51141	−13.3	0.08957	0.70575 ± 3	0.70520	+17
RC 721	Cluanies	Granodiorite	417	2.896	12.46	0.1405	0.512548 ± 37	0.51216	+1.3	0.2152	0.70602 ± 4	0.70474	+10
RC 1282	Ross of Mull	Granite	414	4.219	18.20	0.1402	0.511985 ± 18	0.51161	−9.7	0.9525	0.71201 ± 5	0.70639	+34
RC 976	Bonar Bridge	Granite	400	2.430	9.703	0.1514	0.512169 ± 16	0.51177	−6.8	0.4453	0.70847 ± 7	0.70593	+27
RC 872	Hill of Fare	Granite	413	5.105	25.51	0.1210	0.512339 ± 31	0.51201	−1.8			0.7057*	+24
RC 723	Strath Ossian	Granodiorite	400	5.237	23.33	0.1357	0.512151 ± 20	0.51180	−6.3			0.7060*	+28
BC 14	Aberdeen	Granite	440	11.44	57.08	0.1212	0.511766 ± 41	0.51142	−12.7	0.7524	0.71623 ± 5	0.71151	+107
RC 722	Findhorn	Granite	443	2.592	11.14	0.1407	0.511930 ± 23	0.51152	−10.6	1.104	0.72489 ± 4	0.71792	+198
RC 982	Lochnagar	Granite	415	5.055	30.21	0.1012	0.512236 ± 22	0.51196	−2.7	1.082	0.71269 ± 3	0.70630	+33
GH 18	Garabai Hill	Granodiorite	406	3.797	19.63	0.1169	0.512232 ± 30	0.51192	−3.7	0.3081	0.70956 ± 3	0.70778	+54
RC 724	Cruachan	Granodiorite	410	3.844	22.93	0.1013	0.512058 ± 24	0.51179	−6.3	0.5276	0.70783 ± 3	0.70475	+11
80015	Distinkhorn	Granodiorite	390	7.465	37.15	0.1215	0.512512 ± 16	0.51220	+1.3	1.330	0.71219 ± 3	0.70481	+11
70061	Loch Doon	Diorite	408	4.885	25.35	0.1165	0.512372 ± 17	0.51206	−1.0	0.3064	0.70676 ± 2	0.70498	+14
DBL 3	Loch Doon	Granodiorite	408	4.982	26.82	0.1123	0.512338 ± 26	0.51205	−1.4	0.6230	0.70885 ± 3	0.70523	+18
243	Criffell	Granodiorite	397	4.033	28.69	0.08499	0.512326 ± 20	0.51211	−0.4	0.6478	0.70920 ± 3	0.70553	+21
244	Criffell	Granodiorite	397	5.504	38.52	0.08639	0.512317 ± 20	0.51209	−0.6	0.3805	0.70763 ± 3	0.70548	+21
274	Criffell	Granite	397	2.992	16.30	0.1110	0.512255 ± 26	0.51197	−3.1	3.858	0.72866 ± 3	0.70684	+40
55–75	Fleet	Granite	392	7.489	49.77	0.09097	0.512242 ± 22	0.51201	−2.4	2.488	0.72018 ± 3	0.70629	+32
56–72	Fleet	Granite	392	6.013	33.20	0.1095	0.512265 ± 20	0.51198	−2.9	4.139	0.73136 ± 3	0.7083	+60
57–73	Fleet	Granite	392	5.985	32.57	0.1111	0.512261 ± 21	0.51198	−3.0	5.183	0.73638 ± 2	0.7074	+47
RC 1420	S. Uplands	Basic-clast greywacke	400	5.975	31.87	0.1134	0.512473 ± 28	0.51218	+1.1	0.1443	0.70555 ± 6	0.70473	+10
RC 1427	S. Uplands	Acid-clast greywacke	400	5.813	29.54	0.1189	0.512092 ± 19	0.51178	−6.6	1.097	0.71669 ± 2	0.71049	+92
RC 1430	S. Uplands	Acid-clast greywacke	400	4.806	25.29	0.1149	0.512155 ± 26	0.51185	−5.2	0.9055	0.71384 ± 3	0.70872	+67
CH 5	Cheviot	Granite	390	8.469	36.34	0.1409	0.512277 ± 30	0.51192	−4.2	1.380	0.71376 ± 10	0.70610	+29
RC 971	Eskdale	Granite	429	5.688	30.95	0.1111	0.512134 ± 28	0.51182	−5.1			0.7076*	+51

All isotopic analyses were performed using a fully automated V.G. Isomass 54E mass spectrometer except that ($^{87}\text{Sr}/^{86}\text{Sr}$), marked (*) and all $^{87}\text{Rb}/^{86}\text{Sr}$ ratios except those of Garabai Hill and Distinkhorn (which are new determinations) are from refs 8 and 11. Ion exchange techniques were similar to those of O'Nions *et al.*³⁹ $^{143}\text{Nd}/^{144}\text{Nd}$ and $^{87}\text{Sr}/^{86}\text{Sr}$ are normalized to $^{146}\text{Nd}/^{144}\text{Nd} = 0.7219$ and $^{88}\text{Sr}/^{86}\text{Sr} = 8.37521$ respectively. Bulk-Earth parameters used to calculate ϵ values are $^{143}\text{Nd}/^{144}\text{Nd} = 0.512636$, $^{147}\text{Sm}/^{144}\text{Nd} = 0.1967$, $^{87}\text{Sr}/^{86}\text{Sr} = 0.7045$, $^{87}\text{Rb}/^{86}\text{Sr} = 0.0839$. The $^{143}\text{Nd}/^{144}\text{Nd}$ of BCR-1 is 0.512633 ± 12 and $^{87}\text{Sr}/^{86}\text{Sr}$ of NBS 987 is 0.710275 ± 7 on the V.G. Isomass 54E at SURRC.

tal crust have affected the isotopic compositions to the extent that a change in the age of the basement is recognizable across the Grampian Highlands.

Nd isotopic data

Sm–Nd isotopic analyses of samples from 18 plutons, together with three Southern Uplands Lower Palaeozoic sediments, are presented in Table 1. Techniques have been presented elsewhere²⁰ and are summarized in Table 1. Many of the Sr isotopic analyses in Table 1 have been presented before^{8,11} but some are new determinations of higher precision. The initial ϵ_{Nd} data are plotted geographically in Fig. 1 together with those of Hamilton *et al.*⁴.

It is immediately apparent that nearly all the Caledonian granitic magmas had negative initial ϵ_{Nd} values the range being from +1.3 to −13.3. This situation can be compared with that of the coeval I and S type granitoids of the Lachlan fold belt, south-east Australia where the initial ϵ_{Nd} values have been reported as ranging between +4.4 and −9.8 (refs 5, 21). It appears that the range of initial Nd isotopic compositions in the British Caledonian granitoids is displaced to less radiogenic values by ~3 ϵ_{Nd} units. The British Caledonian granites can also be contrasted with the younger granitic magmas from convergent plate margins such as the Sierra Nevada^{2,3}, the Andes¹, and the Transhimalayan Belt²², all of which are of Mesozoic–Tertiary age and which include substantial associated plutonics with high ϵ_{Nd} .

The only British intrusions with (just) positive initial ϵ_{Nd} are Cluanies and Strontian in the Northern Highlands, Kilmelford in the Grampian Highlands and Distinkhorn in the Midland Valley. Very negative initial ϵ_{Nd} values (<−6) are restricted to the Scottish Highlands (Fig. 1). Thus the Nd isotope systematics display parallels with common Pb and U–Pb zircon systematics in showing differences from north to south across northern Britain^{6,9}. In contrast the Sr and O isotope systematics show no such simple geographical zonation^{8,10}. The variation in ϵ_{Nd} could be due to changes in the mantle across Scotland as has been suggested for some of the cogenetic Devonian volcanics²³, greater crustal assimilation in the north or assimilation of differ-

ent kinds of crust. This is best investigated by combining the Nd data with those of other isotopic systems.

Coupled Sm–Nd and U–Pb systematics

In Fig. 2 the ϵ_{Nd} values of the Newer granites for which zircon data are available are plotted. The shaded boxes represent granites with marked inherited zircon components and it is clear that an approximate cutoff of −6 in ϵ_{Nd} generally demarcates the boundary between granites with inherited zircons and those without. Several points arise from this.

(1) As the inherited zircons are unequivocally derived from the continental crust the lower ϵ_{Nd} values are also a function of crustal contamination or melting.

(2) Granites such as Hill of Fare, Lochnagar, Cruachan which contain little or no inherited zircon, but which lie in the Scottish Highlands, also have high ϵ_{Nd} . That is, the lack of inherited zircons is not a function of their loss by restite separation, but of a different source region.

(3) The Aberdeen and Findhorn granites are thought to be >~440 Myr in age^{8,24} and their low ϵ_{Nd} values and inherited zircons reflect a dominance of old crustal components expected from magmas which were emplaced relatively soon after the climax of high-temperature metamorphism in that area. This accords with the findings of Hamilton *et al.*⁴ for the Strichen and Longmanhill intrusions of similar age and setting.

(4) Excluding Findhorn and Aberdeen, a boundary to the limit of ϵ_{Nd} values of <−6 can be placed through the middle of the Grampian Highlands (Fig. 1). Hereafter this line is called the Mid-Grampian Line.

(5) The Strontian Complex lacks inherited zircons in its outer more mafic parts but contains zircons with a marked memory in the central biotite granite^{6,8}. The ϵ_{Nd} values correlate with this in that they are close to bulk Earth in the outer parts but −6.3 in the biotite granite⁴.

Nd–Sr–O isotopic relationships

In Fig. 3, initial ϵ_{Nd} is plotted versus initial ϵ_{Sr} for all the Caledonian granites analysed. There is no correlation between initial ϵ_{Nd} and initial ϵ_{Sr} . The >440-Myr granites plot to the

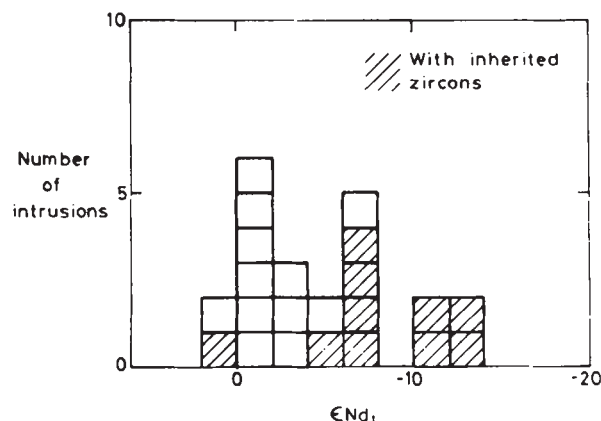


Fig. 2 Histogram of ϵ_{Nd} for all the post-tectonic granite intrusions for which zircon data are available. Shaded boxes are granites with inherited zircons. Data from this study and refs 4, 6, 8.

right with very radiogenic Sr. Moinian or Dalradian assemblages (largely comprising late Proterozoic metasediments) would be suitable crustal components for these magmas^{8,25,26}. Some of the granites with inherited zircons plot towards the field of Lewisian crust. Granitic complexes such as Ross of Mull and Ratagain situated very close to the exposed Lewisian and with low ϵ_{Nd} probably contain a large component of Lewisian crust. However, many of the granites north of the Mid-Grampian Line have exceedingly high Sr contents^{8,27} such that assimilation of Moinian or Dalradian assemblages (with low Sr concentrations) would change the ϵ_{Nd} value towards that of the contaminant more than would be detectable in ϵ_{Sr} . From this reconnaissance study such a process cannot be ruled out.

The Cluanies granodiorite is anomalous in having low ϵ_{Sr} and high ϵ_{Nd} but a marked inherited zircon component. The simplest explanation of this is that it is derived from old light rare-earth elements (LREE)-depleted crust. Some of the more mafic Lewisian granulites might be a suitable source²⁸.

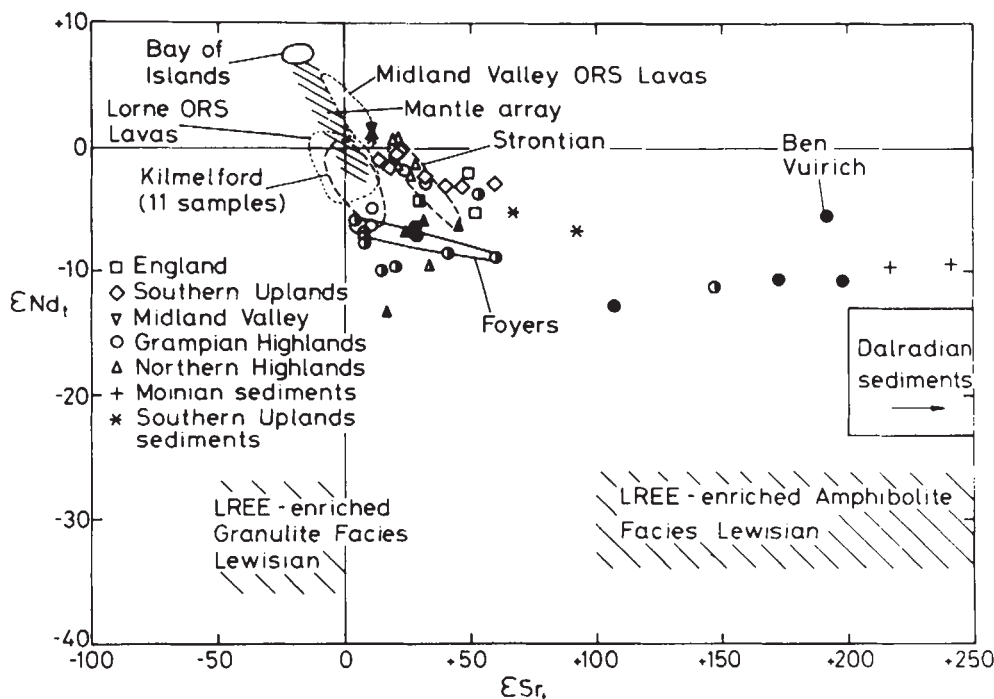
The granites without inherited zircons plot between mantle-like compositions close to bulk Earth and the field of Southern Uplands Lower Palaeozoic sediments. It has been shown elsewhere that these sediments had Pb and Sr isotopic compositions that were similar to many of the Caledonian granites^{8,9,11,41}. They also have the required paucity of Pb isotopic memory in

the zircons such that melting or assimilation of these sediments (unlike those of the Moinian or Dalradian) would not greatly affect the zircon U-Pb systematics in the granites⁸. This is important because there is no doubt that these granites have interacted greatly with the continental crust as shown by their Sr and O isotopic signatures^{10,11}.

In Fig. 4, the initial ϵ_{Nd} values are plotted against $\delta^{18}O$ values given elsewhere^{10,11}. Two samples are ^{18}O -depleted which is thought to be due to high temperature interaction with meteoric waters at their sub-volcanic emplacement level¹⁰. The Helmsdale granite has high $\delta^{18}O$ which has been attributed to late low-temperature fluid interaction²⁹. In contrast the remaining granites have all been regarded as recording magmatic oxygen isotopic compositions, which correlate with initial ϵ_{Sr} but can be seen to be uncorrelated with initial ϵ_{Nd} . However, if the granites with no inherited zircons are considered alone, an inverse correlation is evident—the data points lying between values expected for a depleted mantle source ($\delta^{18}O \sim 6.0\%$, $\epsilon_{Nd} > 0$) and the values for the Southern Uplands acid-clast Lower Palaeozoic sediments. This field of data includes the Strontian granodiorite from the Northern Highlands and samples from Lochnagar, Hill of Fare, Garabal Hill and Distinkhorn—all to the north of the Southern Uplands Lower Palaeozoic sediments. It can be seen that Dalradian metasediments (in which these last granites are situated) are not a major constituent of the source. The field also includes the Southern Uplands granites themselves plus those of the Lake District. The S-type³⁰ granites, Eskdale, Fleet and central Criffell all have high $\delta^{18}O$ ($> 10.0\%$) and slightly lower ϵ_{Nd} (< -2). The outer part of Criffell has higher ϵ_{Nd} consistent with previous hypotheses that the Sr and O isotopic zonation of the pluton is due to interaction with Palaeozoic sediment or its melt derivatives^{11,31-33}. The Shap granite has I-type mineralogy but high $\delta^{18}O$ and ϵ_{Sr} similar to the values for the S-type Eskdale intrusion. The difference in ϵ_{Nd} is consistent with the hypothesis that crustal metasomatic fluids have affected the Shap granite and modified its Sr and O isotopic compositions.

The granites from the north of the Mid-Grampian Line exhibit a limited range of $\delta^{18}O$ and no correlation with ϵ_{Nd} . This is hardly surprising as old high-grade metasedimentary rocks such as comprise the local geology commonly have lower $\delta^{18}O$ values than those of lower grade. Therefore, in general terms, it is to be expected that $\delta^{18}O$ will correlate with $^{87}Sr/^{86}Sr$ as observed because high grade metamorphic rocks will be depleted in Rb as well as having relatively low $\delta^{18}O$. In contrast high-grade

Fig. 3 ϵ_{Nd} versus ϵ_{Sr} plot showing data from the study of Hamilton *et al.*⁴ and this study. Open symbols are for intrusions lacking inherited zircons, filled symbols are for intrusions with a marked inherited zircon memory, half-filled symbols are for intrusions with no U-Pb zircon data. Mantle array is constructed using bulk Earth parameters, the Bay of Islands ophiolite data⁴⁰ and by analogy with the modern mantle array. Other data sources are: Lewisian (ref. 28), Kilmelford (unpublished data), ORS Lavas (ref. 23), Moinian (ref. 25), Dalradian (ref. 8, 26).



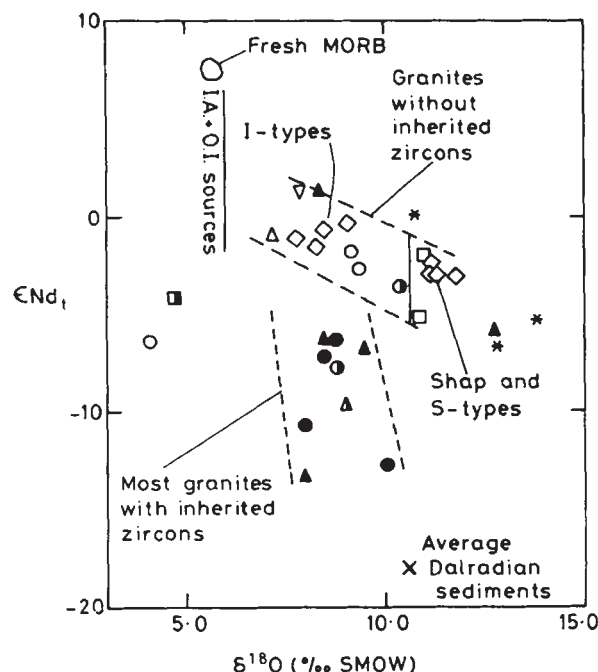


Fig. 4 ϵ_{Nd} versus $\delta^{18}O$. Symbols as in Fig. 3. Data from refs 4, 10, 11 and this study with estimates of fields for mid-ocean ridge basalts (MORB), oceanic islands (OI) and island arcs (IA). Dalradian average is from refs 26, 29.

metamorphism and diagenesis do not readily result in disturbance of U–Pb zircon or Sm–Nd systematics, hence the strong correspondence between those two parameters shown in Fig. 2, but the lack of strong correlation between ϵ_{Nd} and $\delta^{18}O$ or ϵ_{Sr} in the Highland granites (Figs 3 and 4).

The fundamentally important feature of Fig. 4 is that it indicates that granites with marked inherited zircon components and low ϵ_{Nd} have not been more contaminated by the continental crust than granites lacking inherited zircons and with $\epsilon_{Nd} > -6$. They have instead been contaminated by or melted from a different kind of crust, that is, one with markedly older components.

Following on from this it can be seen that the Lochnagar, Hill of Fare and Garabal Hill granitoids, despite having high ϵ_{Nd} and no inherited zircons, have interacted to a major degree with continental crust yielding $\delta^{18}O$ values of $\sim 10\%$. Hence although situated in the Scottish Highlands their lack of inherited zircons and high ϵ_{Nd} do not reflect uncontaminated mantle-derived magmas, but magmas contaminated with young continental crust like that with which the Southern Uplands and English granitoids interacted. U–Pb zircon and Sm–Nd studies of Dalradian^{8,26} lithologies indicate that melting of Dalradian crust would have lowered the ϵ_{Nd} drastically and imparted inherited zircons to the magma (Fig. 4). The data are therefore most simply explained if younger continental crust has been thrust under the southern side of the Grampian Highlands and provided the source for these granite magmas. Finally it should be noted that magmas with a large mantle component could easily plot in the area of intersection of the two fields in Fig. 4 whatever the contaminating crust was like.

Magma genesis in northern Britain

The British late Caledonian granites were clearly not generated by melting of mantle or subducted oceanic crust on their own. The combined data indicate that the continental crust has drastically affected the isotopic characteristics of the magmas. There are certain features of the magmas, however, which indicate that the granites are not simply the product of crustal melting but that many contain a mantle-derived component. These include the abundance of cogenetic high Ni dioritic magmas, the high levels of elements such as Sr (most easily achieved by

differentiation at mantle depths) and the presence of cogenetic basaltic and andesitic lavas²⁷. This, plus the large variation in ϵ_{Nd} observed in a single complex, such as at Kilmelford, suggests that in some cases mantle-derived magmas have assimilated continental crustal melts during their ascent.

In Fig. 3 the fields of data for Devonian mafic lavas from the Midland Valley and Lorne²³ are plotted. The Lorne Lavas are situated in the south-west Grampian Highlands (Fig. 1) and are thought to be cogenetic with the Kilmelford complex. Note that there is an overall downwards displacement of the plutonic data relative to the volcanics. This partly reflects the fact that the data for the volcanics are for high Ni lavas only, whereas the plutonics are largely granodiorites or granites. Hence the more silicic compositions can be (not surprisingly) related to greater interaction with the continental crust. Just as the granites south of the Mid-Grampian Line have higher ϵ_{Nd} than those to the north so also do the Devonian mafic volcanics but displaced overall to higher values (Fig. 3). This parallel behaviour is not regarded as coincidental but is most simply explained by the same process, that is, assimilation of differing ages of crust north and south of the Mid-Grampian Line. This alternative explanation is totally at variance with the mantle heterogeneity model previously invoked to explain the Nd data for the lavas²³.

The data for the lavas do indicate that magmas with 'depleted mantle' isotopic signatures were intruding the continental crust at the time. There is no way that one can assume that the same such magmas were parental to all the intermediate-acid magmatism in the Highlands. It would not be surprising, for example, if the changes in age of continental basement corresponded to changes in the nature of the underlying mantle. What is surprising is that even the diorites from Loch Doon with quite low $\delta^{18}O$ (Fig. 4) have negative ϵ_{Nd} . Unlike those of the Midland Valley these magmas yield no evidence of having been derived from depleted mantle or oceanic crust, which suggests that the Iapetus oceanic crust had been completely consumed by subduction under the Midland Valley by late Silurian times. This is contrary to previous suggestions³⁴. It further requires that the crust underlying the Southern Uplands accretionary prism is allochthonous as suggested elsewhere^{35,36}.

Contamination with older continental crust might explain why the ϵ_{Nd} values of the British granites appear to be displaced by three units relative to the Australian granites. The contaminant clearly varies from Lewisian crust in the far north-west of Scotland to Lower Palaeozoic sediment in the Southern Uplands and Lake District. The exact nature of the basement in between is uncertain. Some of the northerly basement would need to be Lewisian or its sedimentary/magmatic derivatives (Moinian or Dalradian, for example) to produce low ϵ_{Nd} and marked inherited zircons. The southerly basement would need to be like Palaeozoic upper crust further south. Nd and U–Pb zircon data for granulite facies nodules erupted with Midland Valley Carboniferous Lavas indicate a striking similarity with corresponding data for Lower Palaeozoic sediments of the Southern Uplands^{25,37}. A simple way to explain the data for the granites and the granulite facies basement is for the basement to represent Lower Palaeozoic crust thrust northward during Iapetus closure. The view that primary crust of Grenville ($\sim 1,000$ Myr) age is an important crustal component in the Highlands granites³⁸ is difficult to reconcile with the U–Pb zircon evidence. In particular, nearly all of the late Caledonian granites analysed which contain inherited zircons yield data which lie about a reverse discordia with an upper intercept of $\sim 1,600$ Myr (ref. 6). Furthermore neither U–Pb zircon⁸ nor Sm–Nd whole rock²⁶ studies of Scottish Highlands sediments provide evidence for local Grenville-age primary crust. Underthrusting of the crust during the late Caledonian helps to explain the problem of finding a unique crustal component in the Highlands granites^{8,10,29,38} as the crustal structure is likely to have been exceedingly complex. It also provides a partial explanation for the large crustal component in the granites as a whole as juxtaposing crust over hot mantle would be a way of generating granite magma. The simplest interpretation of the data shown

in Fig. 4 is that the southerly crust was largely young accreted material such as arc, trench and oceanic crust fragments.

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Molecular cloning of cDNA for murine interleukin-3

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The cDNA sequence for murine interleukin-3, one of the colony stimulating factors that regulate haematopoiesis, codes for a polypeptide of 166 amino acids including a putative signal peptide. The predicted amino acid sequence indicates that formation of mature interleukin-3 involves proteolytic removal of not only the signal peptide but additional amino-terminal amino acids.

INTERLEUKIN-3 (IL-3) is one of a number of colony stimulating factors which are known to regulate haematopoiesis^{1–3}. The colony stimulating factors are hormone-like glycoproteins which are biologically active at concentrations of 10^{–12} M. They are produced in minute quantities *in vivo* and because of this have proven to be very difficult to characterize by conventional biochemical methods.

IL-3 is produced by mitogen or antigen-activated T lymphocytes and by a number of continuous cell lines^{4,5}. It may also be produced by bone marrow cells. IL-3 is involved in regulating the growth and differentiation of pluripotent stem cells leading to the production of all the major blood cell types. It has a broad range of biological activities and appears to be identical with a number of other factors named on the basis of their biological activities. These include multi-colony stimulating factor CSF, haematopoietic growth factor⁶, burst-promoting activity⁷, P-cell stimulating factor⁸, mast cell growth factor⁹, histamine-producing cell stimulating factor¹⁰ and Thy 1-inducing activity¹¹.

Murine IL-3 derived from the myelomonocytic leukaemia cell line WEHI-3 has recently been purified to homogeneity and a portion of its N-terminal sequence and some of its biochemical and biological properties reported^{12,13}. The protein is glycosylated and the molecular weight (MW) of the major glycosylated species was estimated to be 28,000. A second

species of IL-3 with the same N-terminus was also purified which had an apparent MW of 32,500 suggesting that the two forms may differ in their degree of glycosylation. In the present work we describe translation of IL-3 mRNA in *Xenopus laevis* oocytes and the cloning and sequence analysis of a cDNA coding for murine IL-3.

Translation of IL-3 mRNA in oocytes

Two different murine cell lines were compared as sources of IL-3 mRNA. The myelomonocytic line WEHI-3 is known to produce IL-3 constitutively¹⁴ whereas the lymphoma line EL-4 produces IL-3 after stimulation with phorbol myristate acetate (PMA)⁵. Messenger RNA was prepared from both cell lines using the guanidine thiocyanate method^{15,16} and attempts were made to translate the mRNAs by microinjection into *X. laevis* oocytes¹⁷. The translates were assayed for biologically active IL-3 using the IL-3 dependent cell line 32D cl-23 (ref. 18). It was found that mRNA from EL-4 could be translated in the oocytes to give readily detectable levels of IL-3 after 48 h incubation (Table 1). Most of the detectable activity was present in the incubation medium indicating that the oocytes were secreting mature IL-3. Assays of oocyte extracts showed that they were inhibitory. mRNA from WEHI-3 cells regularly gave higher levels of IL-3 in the translation assays than preparations from EL-4 (Table 1), in keeping with the higher levels of IL-3

Table 1 Translation of IL-3 mRNA in *Xenopus laevis* oocytes

mRNA	Incubation time	IL-3 activity (c.p.m.)		
		Oocyte	Medium	Total extract
EL-4	24 h	0	3,100	3,700
	48 h	—	20,000	5,600
WEHI-3	48 h	6,000	47,000	28,700

EL-4 cells were grown to a cell density of $\sim 10^6$ per ml in spinner cultures in DMEM medium supplemented with 10% fetal calf serum. The cells were collected by centrifugation, resuspended in serum-free medium containing 100 ng ml^{-1} PMA and subcultured into 150 cm^2 culture flasks at 5×10^8 cells per flask. The cells were incubated for 10 h before collection. WEHI-3 cells were grown to a density of $\sim 10^6$ per ml in RPMI-1640 medium supplemented with 10% fetal calf serum and collected by centrifugation. Afterwards, both cell pellets were resuspended in 0.1 volume of serum-free medium and mRNA was prepared by the guanidine thiocyanate-CsCl method^{15,16}. The mRNAs were further purified by chromatography on oligo(dT)-cellulose³² and after ethanol precipitation, were resuspended in buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5) at a concentration of $1 \mu\text{g } \mu\text{l}^{-1}$. The mRNAs were injected into 15 *X. laevis* oocytes ($\sim 50 \text{ nl}$ per oocyte) according to standard methods¹⁷. After incubation, the medium was withdrawn and an extract of the oocytes prepared¹⁷. The incubation medium, the oocyte extract and a mixture of the two were assayed for IL-3 activity using the IL-3 dependent cell line 32D cl-23 (ref. 18) according to the methods described previously, measuring incorporation of ^3H -thymidine as an index of cell growth.

found in culture supernatants of the WEHI-3 cell line (data not shown).

Hybrid plasmids containing IL-3 cDNA

IL-3 mRNA from WEHI-3 was fractionated on a sucrose density gradient and the fractions assayed by oocyte translation (Fig. 1). The major peak of activity sedimented at $\sim 12\text{S}$. This procedure resulted in 10–20-fold purification of the IL-3 mRNA. RNA from the peak fractions was used as template to synthesize double-stranded cDNA using the method of Land *et al.*¹⁹. The resultant cDNA was inserted into pAT153 by G-C tailing²⁰ and the cDNA library transformed into strain HB101. Approximately 5,000 individual clones were isolated. Of a small sample tested, 92% were ampicillin sensitive indicating the presence of cDNA inserts in the majority of the clones. Since the cDNA was not size fractionated prior to cloning, the sizes of inserts in the cDNA library varied from about 200 to 1,000 base pairs (bp). It was expected that even the short inserts would give satisfactory results in the mRNA hybridization-translation assay (see below).

Clones were grown in pools of 10 and DNA prepared by the method of Holmes and Quigley²¹. The plasmids were converted to a linear form with *Bam*HI restriction endonuclease, contaminating RNA was destroyed by alkaline hydrolysis and the DNA was bound to nitrocellulose filters. The filter-bound DNA was hybridized with WEHI-3 mRNA and bound mRNA eluted and translated in oocytes. One positive pool of 10 clones was detected. The activity observed in translates derived from the positive pool was quite low but was consistently above the background and the values of the other clone pools. DNA was therefore prepared from the individual clones of the pool and the mRNA hybridization translation assay repeated. One of the clones (pILM1) was clearly positive (Table 2). The IL-3 activity detected showed the same titration curve as oocyte translates of WEHI-3 mRNA (Fig. 2).

Nucleotide sequence analysis

The cDNA insert in pILM1 was quite short (139 bp excluding the G-C tails). It was subcloned into the *Sma*I site of M13mp8²² after shortening the G-C tails with *Bal*31 exonuclease. The DNA sequence was determined on both strands using the chain termination method^{23,24}. An oligonucleotide primer corresponding to nucleotides 92–112 of this cDNA insert was synthesized^{25,26} and labelled using [γ - ^{32}P]dATP and polynucleotide

Table 2 mRNA hybridization translation assay for detection of IL-3 cDNA clones

DNA sample	IL-3 activity (units per ml)
1	<1
2	<1
3	<1
4	<1
5	<1
6 (pILM1)	121, 106, 139
7	<1
8	<1
9	<1
10	<1

DNA samples ($\sim 30 \mu\text{g}$) were prepared from each clone by the method of Holmes and Quigley²¹. The hybrid plasmid DNA was converted to a linear form by digestion with *Bam*HI restriction endonuclease, extracted with phenol and ethanol precipitated. The DNA was heated in 0.1 M NaOH for 20 min at 70°C , diluted with 20 vol of 2 M NaCl and applied to 1 cm diameter nitrocellulose filters by slow filtration. The filters were washed with $6 \times \text{SSC}$ ($1 \times \text{SSC}$: 0.15 M NaCl, 0.015 M Na citrate, pH 7.0) and baked *in vacuo* at 80°C for 2 h. The 10 membranes were hybridized with 400 μg WEHI-3 mRNA under the conditions described previously³¹ and the bound mRNA eluted according to the procedure of Parnes *et al.*³³. The eluted mRNA was translated in oocytes as described in Table 1. A unit of IL-3 activity is that required to give 50% of the maximum incorporation of ^3H -thymidine in the assay.

kinase²⁷. The labelled probe was used to examine the remaining clones in the cDNA library^{28,29}. No other clones containing this sequence were detected.

To isolate a long clone encoding IL-3, a further cDNA library was prepared, but in this case the double-stranded cDNA was size fractionated using Sepharose CL-4B to enrich for cDNA fragments larger than ~ 500 bp. The resulting cDNA was inserted into pAT153 by G-C tailing and transformed into HB101. The labelled oligonucleotide probe described above was used to identify a longer cDNA clone (pILM3) which was shown by DNA sequencing to carry an insert of 629 bp excluding the G-C tails. The DNA sequence of clone pILM3 was determined on both strands using the chain termination method^{23,24} after subcloning into M13mp8 and 9 (ref. 22). The sequencing strategy is given in Fig. 3a. The subcloning was complicated by the fact that one of the *Pst*I sites flanking the cDNA insert had been lost due to exonucleolytic removal of one nucleotide during the G tailing of the vector. Clone pILM3 was found to extend beyond pILM1 at the 3' end only, both clones possessing the same 5' end. The DNA sequence shows a single large open reading frame (Fig. 3b). Assuming that the first ATG from the 5' end serves as the initiation codon, the reading frame extends from nucleotides 28–525 inclusive and specifies a polypeptide of 166 amino acids. The 5' untranslated region is therefore quite short (27 bp). The method of Land *et al.*¹⁹, which was used to generate the cDNA clones, is designed to produce a high proportion of cDNA clones with complete 5' termini. Since both cDNA clones isolated in the course of this work had the same 5' end, it is possible that pILM3 carries a close to full length copy of the 5' untranslated region of IL-3 mRNA. Clone pILM3, however, does not extend to the 3' end of the IL-3 mRNA and is probably missing a significant portion of the 3' untranslated region. The presence of the complete coding sequence for IL-3 is the important feature in the context of the present work.

The reading frame specifies a protein of MW 18,540. The strongly hydrophobic stretch of 9 amino acids (residues 13–21) is indicative of the presence of a signal peptide. In the cases studied to date, the last residue of the signal sequence is invariably an amino acid with a small uncharged side chain. Analysis of the signal peptide region suggests a number of potential sites for cleavage by the signal peptidase. Application of the empirical rules proposed by Von Heijne³⁰ predicts the end of the signal peptide at residue 27 (Ala). This potential cleavage site is shown with an arrow in Fig. 3b.

Table 3 Amino acid composition of murine IL-3

	Predicted	Determined
Phe	5	4.2
Leu	15	16.7
Ile	5	4.8
Met	1	1.2
Val	10	8.3
Ser	13	11.3
Pro	10	7.1
Thr	8	7.1
Ala	3	4.2
Tyr	2	1.8
His	2	2.4
Glu, Gln	12	13.7
Asp, Asn	18	16.1
Lys	9	9.5
Cys	4	ND
Trp	0	ND
Arg	11	9.5
Gly	6	8.3

The amino acid composition data are taken from ref. 13. The composition was originally calculated for a MW of 23,722 and has been normalized to the size of mature IL-3 which is predicted from the DNA sequence. The normalization was done using the values for Glu and Asp. ND, not determined.

The published N-terminal amino acid sequence of murine IL-3^{12,13} is in complete agreement with the reading frame and extends from residues 33 (Asp) to 47 (Val). It seems most unlikely that the signal peptide would end at residue 32 (Arg) and this suggests that the murine IL-3 isolated from the WEHI-3 cell line has been further processed.

The protein sequence of mature processed IL-3 indicates a MW of 15,102 with four potential *N*-glycosylation sites (Asn-X-Ser or Asn-X-Thr). The four sites are underlined in Fig. 3b. One of these is in the region of the published N-terminal sequence and glycosylation at this site would explain the inability to identify the Asn residue during automated Edman degradation. When the amino acid composition figures for murine IL-3 (ref. 13) are normalized to the size predicted by the DNA sequence they give good agreement with the predicted composition (Table 3).

The amino acid sequence of murine IL-3 was compared with the sequences of human interleukin-2 (ref. 31) and human immune interferon³² both of which are also produced by antigen-activated T lymphocytes. No significant sequence homology was detected. Although human IL-2 is of very similar size to IL-3 it carries no potential sites for *N*-glycosylation and does not appear closely related.

Discussion

The demonstration that IL-3 mRNA can be efficiently translated in oocytes to give biologically active factor provides a very sensitive and useful assay for this mRNA. This assay has enabled the identification of a cDNA clone carrying the entire coding sequence for murine IL-3 using the mRNA hybridization translation method.

Sequence analysis of the cloned cDNA has shown that it codes for a polypeptide of 166 amino acids including a signal peptide of possibly 27 amino acids. Mature IL-3 has been purified to homogeneity^{12,13} from the same WEHI-3 cell line as was used as the source of the IL-3 mRNA in the present work. The N-terminal sequence of the mature IL-3 is in complete agreement with the reading frame but starts at residue 33 suggesting an additional proteolytic cleavage has occurred apart from the cleavage of the signal peptide. The proposed proteolytic cleavage could be catalysed by a number of serine-type proteases such as a member of the kallikrein family or plasmin and could provide another level of regulation if processing was required to generate biologically active IL-3. Whether this processing

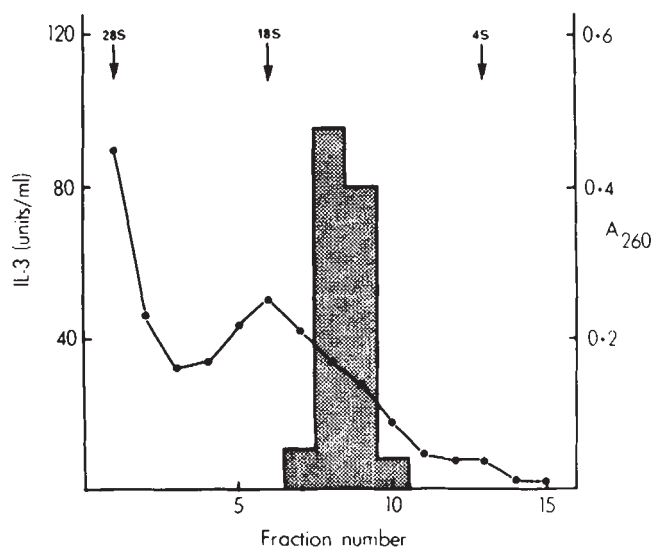


Fig. 1 Sedimentation of IL-3 mRNA through a sucrose density gradient. ●, A_{260} ; hatched areas, IL-3 activity. Poly(A)⁺ mRNA (500 μ g) from WEHI-3 was heated at 80°C for 2 min, chilled in ice and applied to a 5–20% sucrose gradient prepared in a Beckman SW41 polyallomer tube. The gradient was centrifuged at 30,000 r.p.m. for 20 h at 4°C and fractionated into 15 fractions by collection of drops. The fractions were ethanol precipitated and resuspended in 20 μ l of buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 7.5). A portion of the RNA was electrophoresed on a 1.5% agarose gel. The RNA was also translated in *X. laevis* oocytes as described in Table 1. One unit of IL-3 activity is that required to give 50% of the maximum incorporation of ³H-thymidine in the assay.

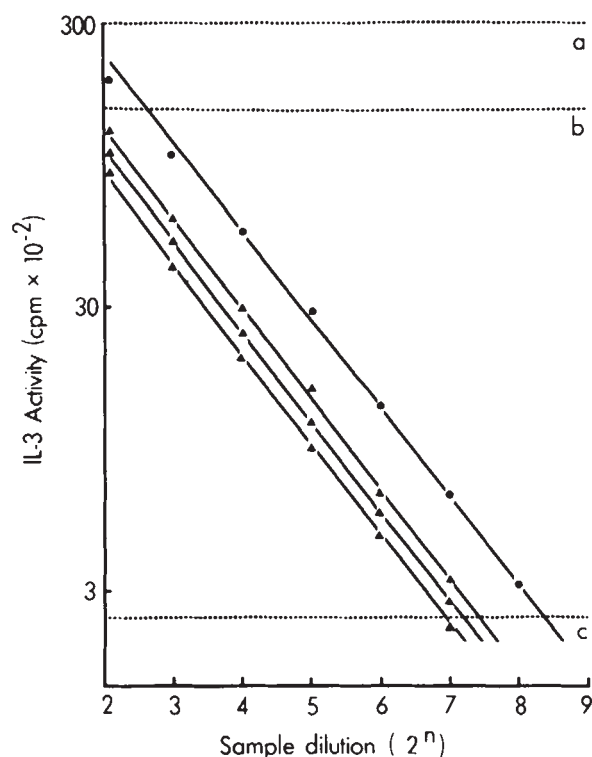


Fig. 2 Titration curves for the IL-3 activity present in oocyte translates. Δ , Translates of mRNA hybridizing to pILM1 (see Table 2); ●, translates of WEHI-3 mRNA. Twofold dilutions of the oocyte translates were made in microtitre trays and the IL-3 activity present was measured using the IL-3 dependent cell line 32D cl-23 as described previously¹⁸. The stimulation of growth of the indicator cells is expressed in terms of ³H-thymidine incorporation. The dotted lines designated a, b and c represent incorporations of 100%, 50% and background plus three standard deviations respectively.

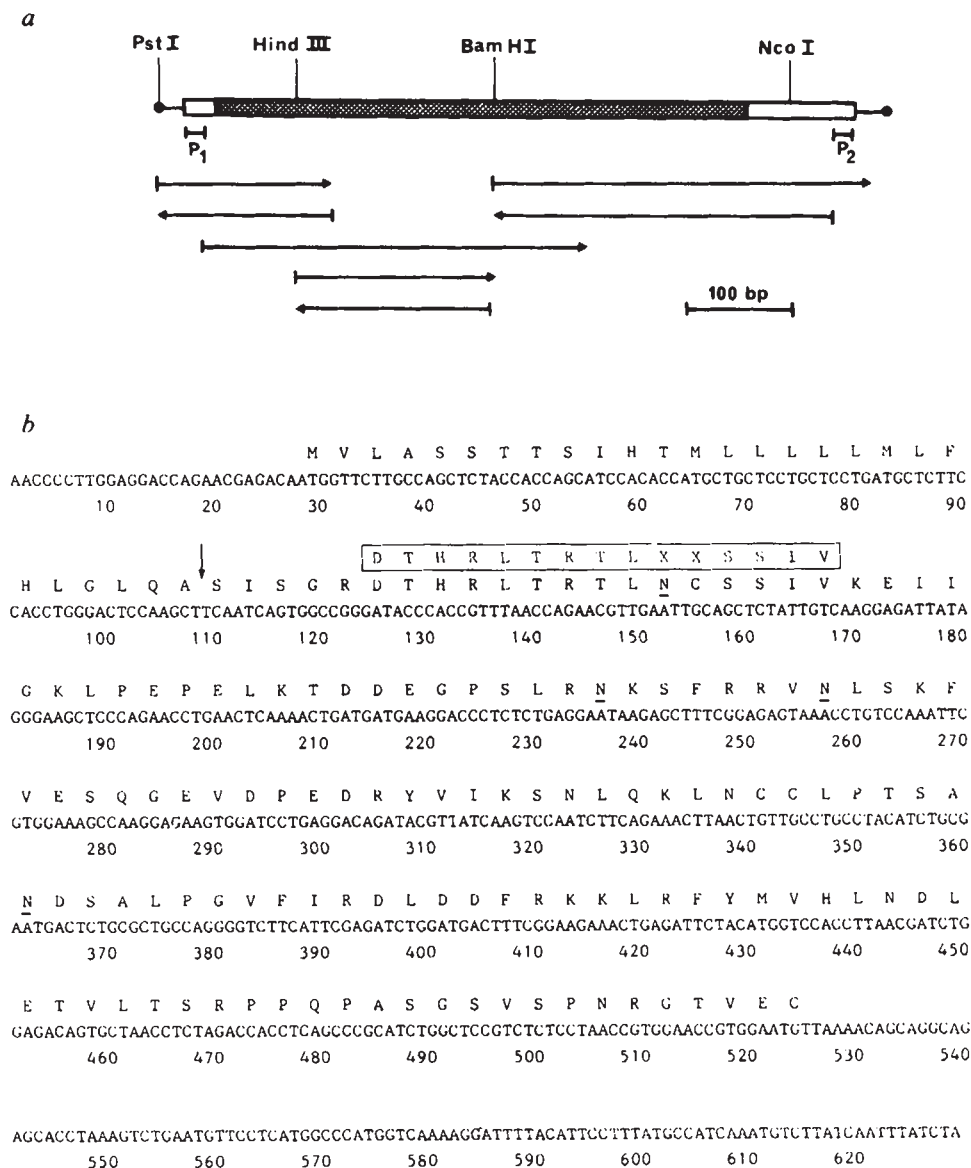


Fig. 3 a, The cDNA insert in pILM3 showing the relevant sites of restriction endonuclease cleavage. The reading frame is delineated from the untranslated regions by hatching and the flanking G-C tails are shown as short bars. One of the expected *Pst*I sites was lost during G-tailing of the vector pAT153. The sequencing strategy is summarized beneath the cDNA insert. DNA sequencing was carried out by the chain termination method^{23,24} after subcloning fragments of pILM3 into M13mp8 and M13mp9²². The computer programs described by Staden^{34,35} were used to assemble and analyse the sequence data. To avoid interference from the longer G-C tails, which was encountered using the universal primer of Duckworth *et al.*³⁶, two internal primers (*P*₁ and *P*₂) 17 nucleotides in length were synthesized^{25,26} and used with the M13 templates. **b**, Nucleotide sequence of the cDNA insert of pILM-3 and predicted amino acid sequence of murine IL-3. The N-terminal sequence of murine IL-3^{12,13} is boxed in above the predicted amino acid sequence. Potential sites for N-glycosylation are underlined. The arrow indicates a possible site of cleavage for removal of the signal peptide³⁰.

step occurs *in vivo* or simply represents adventitious proteolysis during purification is not clear at present.

Apart from the agreement with the N-terminal sequence, the amino acid composition data for mature IL-3 also give good agreement with the predicted sequence. Since it appears that WEHI-3 only produces one member of the group of colony stimulating factors (that is, IL-3), there seems no doubt that the cDNA clone encodes IL-3.

The isolation of a cDNA clone encoding murine IL-3 opens up a number of new molecular approaches to studying the role of this factor in haematopoietic stem cell differentiation. It should prove valuable in studies of proliferative blood disorders such as certain leukaemias and polycythaemias and should enable adequate quantities of murine IL-3 to be produced using suitable expression systems. It should also facilitate isolation of

cDNA clones for human IL-3 and allow investigation of potential applications of this factor in clinical medicine in the area of bone marrow transplantation following cancer chemotherapy.

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Note added in proof: The CDNA encoding IL-3 has now been expressed in monkey COS cells using an SV40 expression vector. The expressed factor supports the growth of the IL-3 dependent cell lines; its full range of biological activities is currently being tested in collaboration with Dr D. Metcalf, Walter and Eliza Hall Institute of Medical Research, Melbourne.

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A molecular map of the human major histocompatibility complex class III region linking complement genes C4, C2 and factor B

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Four human complement genes, which have previously been mapped between HLA-D and HLA-B on chromosome 6, have now been aligned on a 98-kilobase (kb) section of the chromosome on the basis of four overlapping cosmid clones of genomic DNA. The C2 and factor B genes, less than 2 kb apart, are about 30 kb from two C4 genes separated from each other by about 10 kb.

THE major histocompatibility complex (MHC) in man (HLA) is a highly polymorphic region of approximately 2 centimorgans (2×10^6 base pairs, bp) on the short arm of chromosome 6¹. It contains three major classes of genes: class I and class II code for cell-surface glycoproteins which are concerned in self-non-self recognition of cells involved in the immune response, and class III code for some proteins of complement, the major effector system of humoral immunity². There is clinical interest in the HLA not only because the products of the class I and class II genes are the major barrier to tissue transplantation but also because susceptibility to many autoimmune diseases is correlated with the presence of certain haplotypes of class I and II³ and also, because of close linkage⁴, of the class III genes. It has been suggested that the polymorphism of the class III proteins may be a factor in susceptibility to autoimmunity⁵.

The class III genes code for the complement proteins C2, C4 and factor B. C2 and factor B are novel types of serine proteases which, on activation in association with C4 and C3, respectively, form the C3 convertases of the classical pathway (C42) and the alternative pathway (C3B) of complement activation⁶. C2 and factor B are single peptide-chain proteins of approximately 100,000 and 90,000 molecular weight (M_r) while C4 has three disulphide-linked chains, α (95,000 M_r), β (75,000) and γ (30,000), but is synthesized as a single peptide chain of 200,000 M_r (ref. 7).

The structural genes of C4, C2 and factor B have been mapped between HLA-B and HLA-D using protein variants detected mainly by electrophoresis (summarized by Weitkamp and Lamm⁸). C2 and factor B seem to be encoded at single loci but C4 is coded by at least two closely linked loci, C4A and C4B (ref. 9). Four alleles have been recognized in C2 (ref. 10), 11 in factor B¹¹, 13 at C4A and 22 at C4B¹². Null (non-functional) alleles have also been found in C2, C4A and C4B but not so far in factor B⁸.

The recent availability of cDNA probes specific for factor B^{13,14}, C4¹⁵⁻¹⁷ and C2¹⁸ has allowed the isolation of these complement genes. The factor B gene has been isolated and characterized by extensive nucleotide sequence analysis¹⁴. The C4 cDNA probe has been used to characterize partially a C4 gene¹⁵ and to correlate a DNA restriction fragment length polymorphism with the C4 A6 protein phenotype¹⁹.

We have used cDNA probes specific for C4,¹⁵ factor B¹⁴ and C2¹⁸ to map the genes for these three complement proteins in the HLA class III region, by overlapping cloned fragments from a cosmid library of human genomic DNA. The results were confirmed and extended by Southern analysis of whole genomic

DNA. There are at least four class III genes in 98 kilobases (kb) of genomic DNA. There are two C4 loci separated by approximately 10 kb and single factor B and C2 loci which are less than 2 kb apart. The genes are arranged in the order C2, factor B and two C4 genes.

Detection of two C4 genes

The HPFH cosmid library (described in Fig. 2 legend) prepared from genomic DNA extracted from blood cells of a 13-yr-old female, complement typed as C4: A3, A2, B1, B2; factor B: SS; (C2 not determined), was screened with a C4-specific cDNA probe, *Alu-7* (ref. 15). Positive clones were identified and the DNA inserts characterized by restriction mapping. Two cosmid clones, 1E3 and 3A3, were shown together to contain a complete C4 coding sequence as determined by hybridization of Southern blots with a full-length cDNA probe, clone TB13 (probe H) (K. T. Belt, M.C.C. and R.R.P., unpublished). The approximate gene size is 21 kb; however, the exact position of the 5' end of the gene has not been determined. The insert of clone 1E3 also contained 7.5 kb of the 5' end of a second C4 gene approximately 10 kb downstream from the 3' end of the C4z gene. These two genes have been labelled as C4y and C4z as it is not clear which gene represents the C4A and C4B loci.

A comparison by restriction mapping of the 5' ends of the two C4 genes showed that they were similar but that the C4y gene was characterized by 3.3-kb *Bam*HI, 3.5-kb *Kpn*I and 12-kb *Bgl*II fragments, whereas the C4z gene had 4.6-kb *Bam*HI, 12-kb *Kpn*I and 14.5-kb *Bgl*II fragments in the homologous region.

Southern analysis of genomic DNA from a sample of 72 individuals (data not shown), including the donor (HPFH) for the genomic library, suggests these two different forms of C4 gene are common in the population. Figure 3 shows a southern blot of genomic DNA from the library donor digested with *Kpn*I, *Bam*HI, or both and hybridized with a 400-base pair

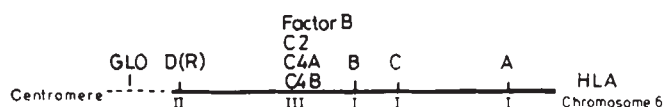


Fig. 1 Map of HLA region on the short arm of human chromosome 6 showing the approximate positions of the class I genes (A, B, C), the class II genes D(R) and the class III genes, factor B, C2, C4A and C4B. The glyoxalase gene (GLO) lies between D(R) and the centromere.

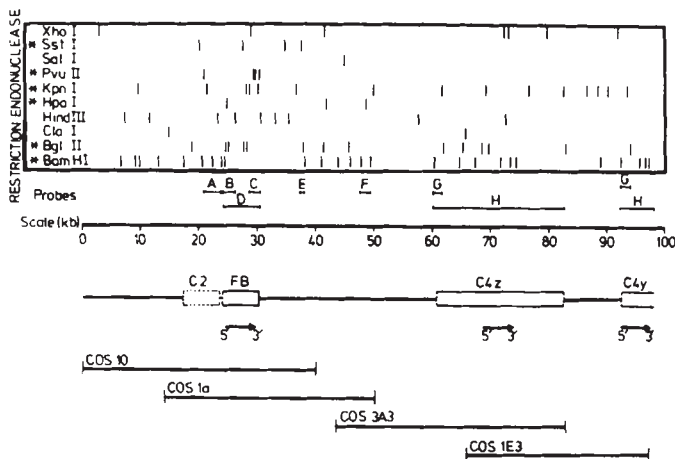


Fig. 2 Molecular map of 98 kb of genomic DNA in the HLA class III region containing four complement genes. Approximately 200,000 colonies from the HPFH cosmid library, prepared by F. Grosveld²⁶ from genomic DNA extracted from white blood cells of a 13-yr-old female, were screened with C4¹⁵ and factor B¹⁴ cDNA probes. DNA inserts from positive clones were characterized further by restriction mapping and Southern analysis using probes A-H as described below. Four cosmid clones 10, 1a, 3A3 and 1E3 were shown to overlap covering a total distance of 98 kb. Probes used: A, 400-bp C2 cDNA, pC201; B, 660 bp *Clal*/*Bam*HI fragment from 5' end of full-length factor B cDNA clone pFB3b; C, 500 bp *Xho*/*Bam*HI fragment from 3' end of full-length factor B cDNA clone, pFB3b; D, 2.4-kb full-length factor B cDNA clone, pFB3b; E, 600-bp *Sst*/*Bam*HI fragment from Cos 1a; F, 1.6-kb *Bam*HI fragment from Cos 3A3; G, 400-bp *Bam*HI/*Kpn*I fragment from 5' end of full-length C4 cDNA clone TB13; H, 5.4-kb full-length C4 cDNA clone.

Methods: Colonies were plated onto 16 nitrocellulose filters (14 cm Millipore HAWP, 0.45 μ M) on nutrient agar plates with ampicillin. Replicas were prepared and processed for colony hybridization according to Grosveld *et al.*²⁷. Prehybridization and hybridization conditions, solutions and procedure for nick translation of probes are described in Fig. 3 legend. After hybridization, filters were washed with four changes of 0.3 M NaCl/0.03 M sodium citrate at room temperature, four changes of 0.15 M NaCl/0.015 M sodium citrate at 65 °C over 2 h and finally with two changes of 0.03 M NaCl/3 mM sodium citrate at 65 °C for 30 min each. Filters were dried and autoradiographed at -70 °C for 1-2 days. Cosmid DNA was extracted from bacterial colonies by using the alkaline SDS method²⁸ and the inserts characterized by single and double digestion with various restriction endonucleases using the manufacturer's specified conditions. Separation of restriction fragments by agarose gel electrophoresis and transfer to nitrocellulose filters for Southern analysis was performed as described in Fig. 3 legend. Positioning of the four genes was based on hybridization with the respective cDNA probes. As the probes (all but E and F) were for coding sequence, the precise limits of the genes were only defined for the 3' end of the factor B gene¹⁴. Orientation of gene transcription for factor B, C4z and C4y was determined by hybridization with cDNA probes specific for 5' and 3' ends.

(bp) C4 cDNA probe, G, a *Bam*HI/*Kpn*I fragment from the 5' end of full-length cDNA clone TB13 (probe H). Both forms of the C4 gene are seen as characterized by 3.3-kb *Bam*HI and 3.5-kb *Kpn*I for C4y and 4.6-kb *Bam*HI and 12-kb *Kpn*I bands for C4z. Only a single hybridizing band of 0.8 kb is seen after double digestion with *Kpn*I and *Bam*HI, suggesting that the two genes are similar in this region. It is not known if these restriction site differences are representative of coding sequence differences.

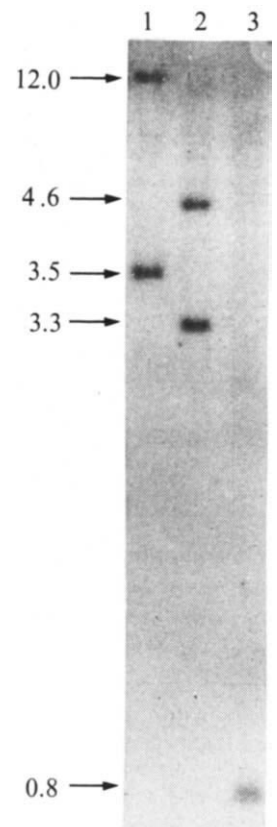
Preliminary characterization of a third cosmid clone which possibly overlaps clone 1E3 but not 3A3 (data not shown) suggests that a third C4 gene may be present and this is being investigated further (M.C.C., unpublished observation).

A single factor B gene

Initial screening of 200,000 clones of the HPFH cosmid library using the 515-bp factor B-specific cDNA probe, FBI¹⁴, resulted in the isolation of one positive clone (cos 1a) with an insert of

Fig. 3 Two common forms of C4 gene in genomic DNA. Southern²⁹ analysis of *Kpn*I (track 1), *Bam*HI (track 2) or *Kpn*I/*Bam*HI (track 3) digests of genomic DNA from individual HPFH hybridized with a 400-bp cDNA probe G, specific for 5' coding sequence of a full-length cDNA TB13, probe H (see Fig. 2 for position of probes). The 3.3-kb *Bam*HI and 3.5-kb *Kpn*I bands are characteristic of C4y, whereas the 4.6-kb *Bam*HI and 12-kb *Kpn*I bands are characteristic of the C4z gene. The single 0.8-kb *Kpn*I/*Bam*HI band in track 3 is common to both C4y and C4z genes.

Methods: Genomic DNA was prepared from whole blood by the procedure of B. Sykes (unpublished). Briefly, after lysis of cells in 0.9% NaCl/0.1% Nonidet P40, pellets were broken up in lysis buffer (7 M urea, 0.3 M NaCl, 10 mM EDTA, 10 mM Tris-HCl pH 7.5) with a glass rod, and incubated at 37 °C for 10 min after the addition of 2% SDS. DNA was extracted 3 $\times$ with phenol/isoamyl alcohol/chloroform (45:10:45) followed by precipitation by ethanol and dialysis against 10 mM Tris-HCl pH 7.5, 1 mM EDTA. For Southern analysis, 8-10 μ g DNA samples were digested for 20 h at 37 °C in a final volume of 50 μ l with 18 U of the respective restriction endonuclease according to the manufacturer's specifications (Boehringer Mannheim), electrophoresed in 0.8% agarose and transferred to nitrocellulose filters (Sartorius). Nitrocellulose filters were prehybridized at 42 °C for at least 2 h in 50% formamide, 1 M NaCl, 0.01 M EDTA, 10 $\times$ Denhardt's solution, 10% dextran sulphate, 50 mM Tris-HCl pH 7.5, 0.1% sodium pyrophosphate and 0.1 mg ml⁻¹ of heat-denatured herring sperm DNA³⁰ and then hybridized with nick-translated (Amersham International)³¹, heat-denatured DNA probes with a specific activity of 10⁸ c.p.m. μ g⁻¹ (0.5 μ Ci ml⁻¹) for 24-48 h. After hybridization, filters were washed six times for 10 min in 2 $\times$ SSC, 0.1% SDS (1 $\times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate) and six times for 10 min at 68 °C in 1 $\times$ SSC, 0.1% SDS. After drying, filters were exposed to X-ray film with an intensifying screen at -70 °C for 2 days. Sizes of all bands were estimated on the basis of radioactive size markers (not shown).



approximately 35 kb. To generate overlapping clones and extend the region of DNA, a 600-bp *Bam*HI/*Sst*I fragment, probe E (Fig. 2), lying 12 kb from the 3' end of cos 1a was used as a hybridization probe of the library. A further six positive clones were isolated and characterized. Four appeared to be identical with cos 1a, while two (cos 10 and cos 16) extended cos 1a by 14 kb at the 5' end (Fig. 2).

Extensive mapping and Southern blot analysis of the cloned genomic DNA using a 2.4-kb full-length cDNA probe, D (ref. 20), showed the presence of only one factor B gene within this region (Fig. 2). The probe hybridized to adjacent 2.7- and 4.4-kb *Hind*III fragments, and sequence analysis¹⁴ has shown that the 3' end of the gene lies approximately 150 bp from the 3' end of the 4.4-kb fragment (Fig. 2). The 5' end of the gene was mapped using an 81-bp cDNA probe, generated from the full-length clone by a *Bam*HI/*Sau*3A double digest²⁰. The probe, which contained coding information for 9 amino acids at the N-terminus of Ba and 10 amino acids of a putative leader peptide, hybridized to a 600-bp *Bam*HI fragment contained within the 2.7-kb *Hind*III fragment (Fig. 2). The 3' end of the *Bam*HI fragment was shown by sequence analysis to encode the N-terminus of Ba and four amino acids of the leader peptide, before running into an intron. Thus, the region that codes for the mature factor B protein is contained within 5.7 kb of DNA.

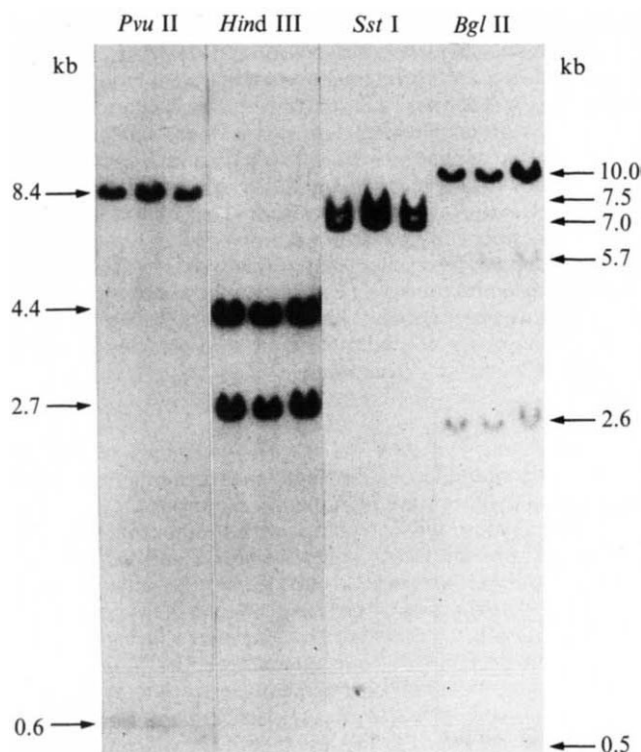


Fig. 4 Southern blot analysis of genomic DNA from three unrelated individuals with the 2.4-kb factor B cDNA probe, pFB3b. 10 μ g of DNA was digested to completion with the enzyme indicated, separated on a 0.7% agarose gel and transferred to nitrocellulose filters as described in Fig. 3 legend. The insert of clone pFB3b, probe D (see Fig. 2), was excised using a *Clal/Bam*HI double digest, and the 660-bp and 1.6-kb fragments generated were purified by elution from a polyacrylamide gel. The fragments were mixed in equal ratio and nick translated to a specific activity of $0.4\text{--}1 \times 10^8$ c.p.m. per μ g DNA such that the hybridization mix contained $\sim 5 \times 10^5$ c.p.m. per ml. Conditions for hybridization, washing and autoradiography were as described in Fig. 3. Unequal intensity of 10-kb and 2.6-kb *Bgl*II fragments is due to unequal labelling of the mixed probes on nick translation.

Due to the close linkage of the factor B and C2 genes (see below), it is likely that the regions responsible for the control of factor B expression lie within the 600-bp *Bam*HI fragment, suggesting that the complete gene is about 6 kb long.

Southern blot analysis of various digests of genomic DNA from three unrelated individuals (Fig. 4) showed that in all cases the sizes of the restriction fragments hybridizing to the factor B probe (D) were as predicted from the map in Fig. 2. The results of these and other digests (*Kpn*I, *Bam*HI, *Acc*I, *Eco*RI, data not shown) indicate the presence of a single factor B gene per chromosome.

The C4 and factor B genes are linked

DNA inserts from cosmid clones 3A3 (containing the C4 gene) and 1a (containing the factor B gene) were shown to overlap by approximately 6 kb. Complementary sequence in this region was further proven by preparing a probe from clone 3A3 in the region of overlap. A 1.6-kb *Bam*HI genomic fragment (probe F, see Fig. 2) was nick translated and hybridized to a Southern blot comparing clones 3A3 and 1a. Probe F from clone 3A3 hybridized to the common restriction fragments in clone 1a, proving that the cloned inserts were complementary in the region of restriction site overlap.

To prove that these class III genes exist in the same order in uncloned genomic DNA, the two cosmid probes F and E (see Fig. 2) isolated from clones 3A3 and 1a, respectively, were hybridized to Southern blots of genomic DNA digested with either the single enzyme *Kpn*I or double digested with both *Kpn*I and *Hpa*I. As shown in Fig. 5a, both probes hybridized

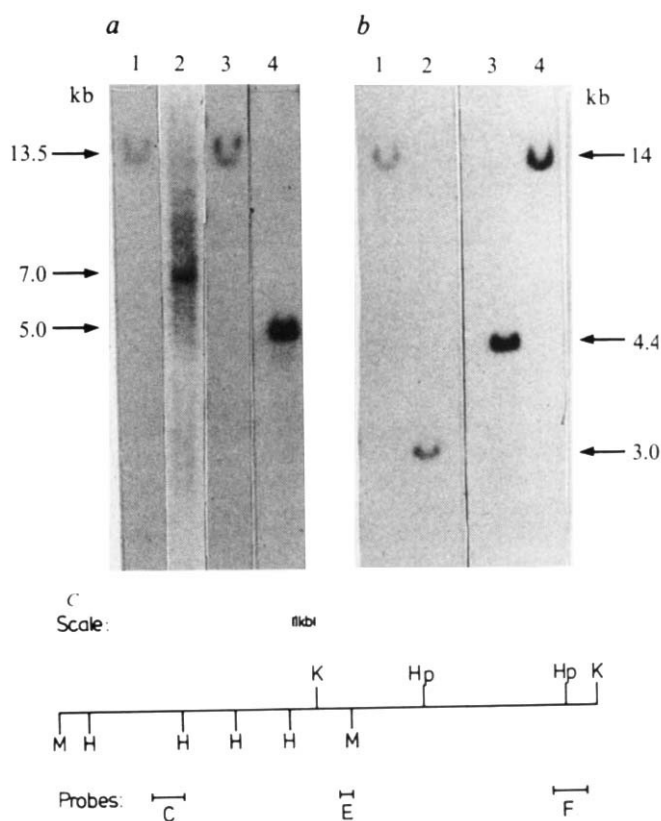


Fig. 5 Genomic probes from cosmid clones containing C4 and factor B genes hybridize to a common genomic restriction fragment. **a**, Genomic DNA was digested with restriction endonuclease *Kpn*I only (tracks 1, 3) or both *Kpn*I and *Hpa*I (tracks 2, 4) and analysed on Southern blots as described in Fig. 3 legend. After hybridizing first with probe F (tracks 1, 2) from cos 3A3 (containing the C4 gene), the filter was treated with 0.5 M NaOH to remove the first probe, neutralized and prehybridized as described in Fig. 3 legend, then hybridized with probe E (tracks 3, 4) from cos 1a (containing the factor B gene). The 13.5-kb *Kpn*I fragment in tracks 1 and 3 hybridized with both genomic probes. After double digestion with *Kpn*I and *Hpa*I shown in tracks 2 and 4, the 13.5-kb *Kpn*I fragment is split into 7- and 5-kb fragments which hybridize with probes F and E, respectively. The 1.5-kb *Kpn*I/*Hpa*I fragment is not seen, because fragments of that size electrophoresed off the gel. For illustration, tracks 1 and 3 from one filter were combined with tracks 2 and 4 from a second filter. **b**, Genomic DNA was digested with *Bam*HI (tracks 1, 4) or both *Bam*HI and *Hind*III (tracks 2, 3) and analysed on Southern blots (see Fig. 3 legend). Samples 1 and 2 were hybridized with genomic probe E from cos 1a while samples 3 and 4 were hybridized with the cDNA probe C (see Fig. 2 legend). Both probes hybridized to a common 14-kb *Bam*HI fragment (tracks 1, 4). After double digestion with *Bam*HI and *Hind*III, probe E hybridized to a 3-kb fragment (track 2) while probe C hybridized to a 4.4-kb fragment (track 3) as predicted from the map in Fig. 2. **c**, Line diagram of restriction map covering region of overlap between cos 3A3 and 1a and factor B gene. Restriction enzymes used: K, *Kpn*I; Hp, *Hpa*I; M, *Bam*HI; H, *Hind*III.

to the 13.5-kb *Kpn*I fragment. The double digest with *Kpn*I and *Hpa*I gave two fragments, a 7-kb band which hybridized only with probe F, and a 5-kb band which hybridized only with probe E. Second, the two probes E and C were hybridized to blots of genomic DNA digested with *Bam*HI only, or *Bam*HI and *Hind*III. Both hybridized to a common 14-kb *Bam*HI fragment (Fig. 5b, tracks 1, 4). When the DNA was digested with *Bam*HI and *Hind*III, probe E hybridized only to a 3-kb fragment while probe C hybridized to a 4.4-kb fragment. Linkage of genomic probe F with probe G, a C4 cDNA probe, was demonstrated by hybridizing to a common 14.5 *Bgl*II fragment (not shown). These results of Southern blots of genomic DNA confirmed the linkage of the C4z gene to the factor B gene proposed from the results of the cosmid mapping.

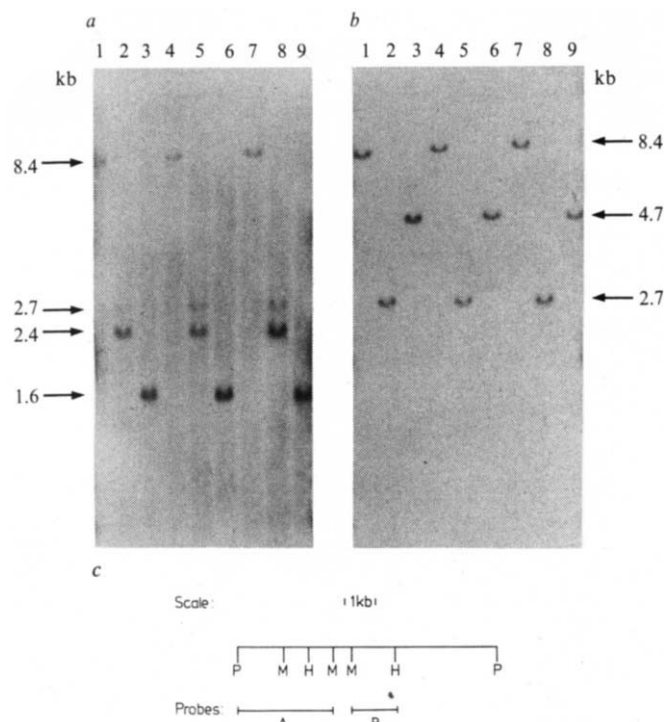


Fig. 6 *a, b*, Southern blotting analysis of genomic DNA from three unrelated individuals, KT, PR and AB using the C2 probe A (shown in *a*), or the Ba probe B (shown in *b*). *c*, Summary of the restriction map in the region of genomic DNA for which the linkage between the two probes was demonstrated. The restriction enzymes used were as follows: tracks 1, 4, 7: *Pvu*II; tracks 2, 5, 8, *Pvu*II and *Hind*III; tracks 3, 6, 9, *Pvu*II and *Bam*HI. The C2 and factor B probes both hybridize to a common 8.4-kb *Pvu*II fragment (tracks 1, 4, 7 in *a* and *b*). When the DNA was simultaneously digested with *Pvu*II and *Hind*III, probe A hybridized to two fragments of 2.4 kb and 2.7 kb (tracks 2, 5, 8 in *a*) while probe B hybridized only to the 2.7-kb fragment (tracks 2, 5, 7 in *b*). Both probes hybridized to the 2.7-kb *Hind*III fragment as the 3' end of the C2 gene and the 5' end of the factor B gene lie within this fragment. Similarly, using DNA digested with *Pvu*II and *Bam*HI, probe A hybridized to fragments of 1.6 kb (tracks 3, 6, 9 in *a*) while probe B hybridized to a 4.7-kb fragment (tracks 3, 6, 9 in *b*) as predicted from the restriction map in Fig. 2.

Methods: 20 µg of DNA was digested to completion with the appropriate one or two restriction enzymes. The reaction mixture was divided into two equal aliquots and one was loaded onto each of two 0.7% 20 cm × 20 cm agarose gels. Each was electrophoresed in parallel and the DNA blotted onto nitrocellulose as described in detail in Fig. 3 legend. DNA fragments used as probes were purified by elution from polyacrylamide thin gels and then nick-translated to a specific activity of $0.2\text{--}1.0 \times 10^8$ c.p.m. per µg DNA. Conditions for hybridization, washing and autoradiography were as described in Fig. 3 legend.

Linkage of C2 and factor B genes

A C2-specific 400-bp cDNA probe, pC201, has been isolated that contains sequence coding for a short region of the C2 protein near its C-terminus¹⁸. The pC201 probe, probe A, was used to identify the position of the C2 locus relative to the genes for C4 and factor B in the cosmid clones already isolated. Probe A hybridized to restriction fragments derived from cos 1a and cos 10, but not 3A3 (results not shown), and the probe site was mapped to within a 2.6-kb region of cos 1a (Fig. 2) that lies approximately 0.5 kb from the 5' end of the Ba coding sequence. The approximate position of the C2 gene is therefore as indicated by the dotted lines in Fig. 2. Confirmation of the close linkage of the C2 and factor B genes was provided by Southern analysis of genomic DNA of three unrelated individuals using probe A, and a factor B probe that was specific for the 5' end of Ba (probe B in Fig. 2). The results showed that the C2 (A) and factor B (B) probes hybridize to a common 8.4-kb *Pvu*II frag-

ment (tracks 1, 4, 7, Fig. 6a, b). When the genomic DNA was simultaneously digested with *Pvu*II and *Hind*III, probe A hybridized to a 2.4-kb fragment and more weakly to a 2.7-kb fragment (Fig. 6a, tracks 2, 5, 8), while probe B hybridized only to the 2.7-kb fragment (Fig. 6b, tracks 2, 5, 8). Similarly, using DNA digested simultaneously with *Pvu*II and *Bam*HI, probe A hybridized to two fragments of 1.6 kb while probe B hybridized to a 4.7-kb fragment (compare Fig. 6a and b, lanes 3, 6 and 9). These results, which are summarized in Fig. 6c, agree with the detailed restriction map already determined for this region of chromosome 6 (see Fig. 2), and therefore confirm that the C2 locus maps within 2 kb of the 5' end of the factor B gene. These results also indicate that the C2 protein is encoded by a single locus per chromosome.

Discussion

A molecular map of 98 kb of the HLA class III region was prepared by ordering overlapping cloned genomic fragments. Four class III genes were identified in the order C2, factor B, C4a, C4y. These results, combined with results from Southern analysis of genomic DNA, suggests that C2 and factor B are encoded by single loci and C4 is encoded by at least two separate loci. While both forms of C4 gene are common in the population, further analysis will be necessary to determine which genes code for the C4A and C4B serum proteins.

Finding that these four complement genes are located within such a relatively close distance of each other, that is, 30 kb between C4 and factor B and less than 2 kb between factor B and C2, explains the failure to observe cross-overs²¹. It is most likely that the C2 and factor B genes evolved from a common locus as they are very close and the proteins have a similar structure and function. C4 has a function related to but distinct from that of C2 and factor B and although the full amino acid sequence of factor B^{20,22} and about a quarter of that of C4 is available²³ there is no obvious similarity. The class I and class II molecules are related in structure to each other (and to the immunoglobulins) and there therefore appears to be at least three distinct types of protein encoded in the MHC, represented by: (1) class I and class II proteins; (2) C2 and factor B; (3) C4A and C4B. The 21-hydroxylase gene has been mapped close to the class III genes in HLA⁸ and the neuraminidase gene to class III genes in the H-2 in mice²⁴ but little information is available on the structures of the corresponding proteins. Their catalytic function seems to be quite unrelated to any of the roles in immunity of the three main classes in the MHC. There is room for more genes between *HLA-D* and *HLA-B*; we hope to identify any coding sequences that may be present and discover whether they are related structurally or functionally to the class I and II or class III genes.

The gene order factor B, C4B, C4A and C2 with C2 nearest the *HLA-B* locus has been proposed by Olaisen *et al.*²⁵ on the basis of haplotypes existing in the population. Our results corroborate their estimate of the distance between factor B and C4 as 0.04 centimorgans (assuming 1 centimorgan = 10^6 bp), but the order of the genes determined in our analysis places the C2 locus next to factor B, not on the opposite side of the C4 genes.

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Transcriptional interference in avian retroviruses—implications for the promoter insertion model of leukaemogenesis

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The downstream (3') long terminal repeat (LTR) of an avian retroviral provirus is unable to act as an efficient promoter of transcription when a transcriptionally active upstream (5') LTR is present. This transcriptional interference may explain the observation that only deleted proviruses have been observed inserted adjacent to c-myc in avian leukosis virus induced lymphomas of chickens.

AVIAN leukosis viruses (ALVs) cause neoplastic disease in their hosts only after a long latent period and cannot be shown to cause transformation of cultured cells *in vitro*. This delayed transformation is thus markedly different from that caused by the acutely transforming retroviruses which have been shown to contain oncogenes encoding transforming proteins¹.

Integration of an ALV provirus into the host DNA results in the viral coding sequence being flanked by two long terminal repeats (LTRs)^{2,3} (Fig. 1A). The viral LTRs contain sequences required for initiation as well as for polyadenylation of viral transcripts and fulfill these roles at the opposite ends of the proviral transcriptional unit⁴. As the proviral LTRs are identical it is possible that the downstream LTR can also act as a transcriptional promoter. The 'promoter insertion' theory (Fig. 1A) predicts that ALV causes neoplasms when this downstream LTR transcribes elevated levels of an adjacent *onc* gene analogous to those observed in the acutely transforming viruses⁵.

Evidence for promoter insertion oncogenesis has been presented by several workers for lymphomas induced by ALV in chickens⁵⁻⁷. Transcripts have been observed containing the U5 region of the ALV LTR and the coding sequence of *c-myc*, the cellular homologue of the transforming gene *v-myc* of the acutely transforming virus MC29. An unexpected result of this research was the finding that the proviruses integrated into the region 5' of the *c-myc* gene were always defective⁵⁻⁷. The observed deletions encompassed different parts of the viral coding region but always appeared to delete the upstream or 5' LTR. Two theories have been advanced to account for this observed defectiveness⁵⁻⁷. One suggestion is that the production of viral gene products by the provirus in a tumour would render it susceptible to detection by the host immune system. However, this appears unlikely as many tumours actively express viral genes encoded by other intact proviruses^{5,6}. A second possibility arises from the structure of the LTR (Fig. 2). When the LTR functions as a promoter, RNA polymerase is thought to interact with sequences in the U3 region of the LTR before initiating transcription at the 'CAP' site. ALV mRNAs are polyadenylated at a site 21 bases 3' to the 'CAP' site⁸. RNA polymerases terminating at this site in the 3' LTR must transcribe the entire viral U3 region in addition to the 21 bases shared by any initiating transcript (the R region). The observation that

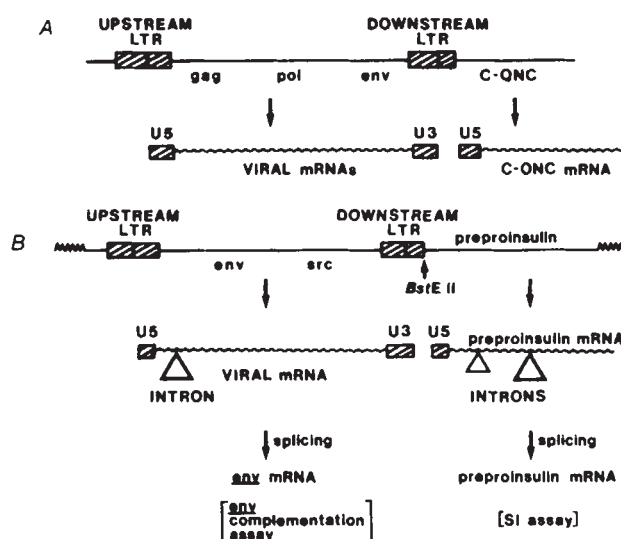


Fig. 1 Experimental strategy for measurement of transcriptional interference. A, Expected structure and transcriptional products of an ALV provirus integrated adjacent to a cellular *onc* gene; B, Structure of clone pBC81.

Methods: The proviral region of the clone was constructed as previously described from Schmidt-Ruppin B sequences. The upstream (5') LTR and *env* gene splice donor site were obtained from clone λ SRBtd8²⁸. They were joined as previously described¹² to a SR-B DNA fragment from clone pLD6 (D. Stacey, unpublished results) which contains the *env* gene splice acceptor site, the *env* gene, the *src* gene and the downstream LTR. This construction generates a provirus-like structure which is deleted for the majority of the *gag-pol* region of the virus which forms the *env* gene intron. We have previously demonstrated that this deletion does not affect *env* gene transcription and splicing¹¹. The rat preproinsulin II gene used contains a *Hind*III site inserted in the 5' non-coding region as previously described¹³. To place the preproinsulin II gene under downstream (3') LTR control, a *Bst* EI site at the end of the U5 region of the downstream LTR was converted to a *Hind*III site. This construction adds an additional 107 bp of 5' non-coding sequences of retroviral origin to the preproinsulin II transcript.

Table 1 Relative *env* and proinsulin mRNA levels from transfected cells

Lane	Clone	<i>env</i> activity		Insulin mRNA synthesis			
		FFU produced*	Per cent	c.p.m. per band	Per cent	Peak area	Per cent
2	pBC8IΔ3	0	0	366	54	237	70
3	pBC8IΔ2	425	0.6	676	100	340	100
4	pBC8IΔ1	52,500	79	225	33	122	36
5	pBC8I	66,000	100	126	19	73	21
9	pBC8I/PA	250	1.3	536	100	306	100
10	pBC8I	18,500	100	149	28	62	20
11	pBC8IΔ3	0	0	287	54	200	66

env activity is shown as the total number of focus forming units produced per transfected culture and as a percentage of the activity of clone pBC8I. Lane nos refer to Fig. 3. Relative insulin mRNA transcription was determined by two methods: peak areas derived by densitometry tracing of the autoradiograph shown using a Beckman DU-8 scanning spectrophotometer, and liquid scintillation counting of actual gel slices from the region of the 170-nucleotide fragment in each lane (values corrected for background counts = 193 c.p.m.). These two measures of insulin mRNA activity are expressed as a percentage of the activity of clone pBC8IΔ2 or pBC8I/PA.

* FFU, focus forming unit.

transcripts extending beyond the 3' end of the mature mRNA are synthesized during transcription of both retroviral⁹ and cellular genes¹⁰ suggests that there is an extensive overlap between the regions involved in initiating and terminating transcription (Fig. 2A). This overlap could reduce the ability of the downstream or 3' LTR to act as an efficient promoter by interfering with initiation of transcription. This inhibition, which we term transcriptional overlap interference, would be relieved by the deletion of the upstream LTR. This deletion may be required if the downstream LTR is to promote sufficient *c-onc* transcription to cause transformation.

Experimental approach

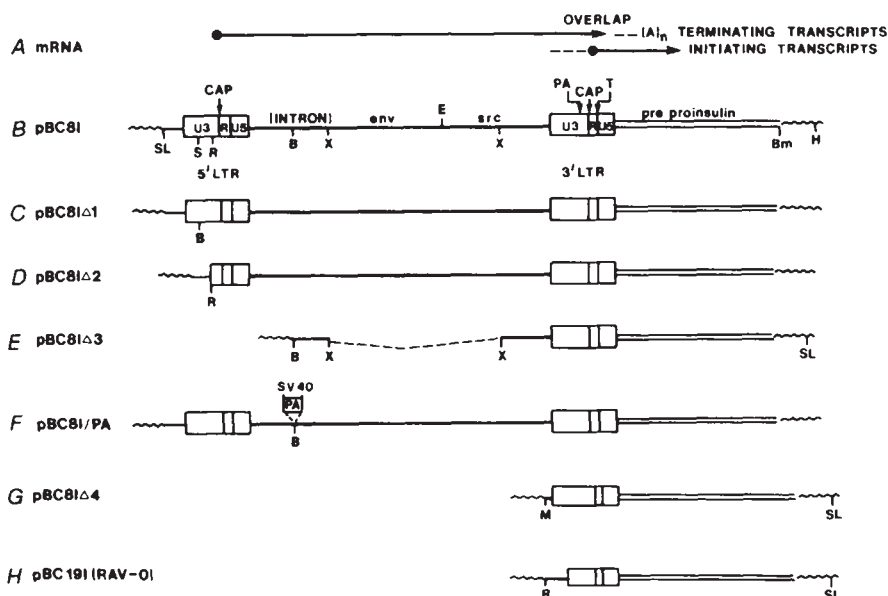
To demonstrate transcriptional overlap interference we have constructed proviral clones in which the upstream and downstream LTRs control the transcription of genes whose levels can be measured independently after transfection into avian cells. The parent plasmid used, pBC8I, is closely analogous to the proposed structure observed after integration of a provirus

next to a cellular *onc* gene⁵ (Fig. 1B). The gene whose transcription is controlled by the upstream LTR is the viral *env* gene whose transcript terminates, as in the proviral case, in the downstream LTR. The gene transcribed under the control of the downstream LTR could, in theory, be any cellular gene. The gene we have chosen to use is the rat preproinsulin II gene. We have previously described sensitive assays for both the viral *env* and the rat preproinsulin II genes¹¹⁻¹³.

The *env* assay is based on complementation of a genetic deficiency in a virus infecting the quail cell line QCI-3. These cells are transformed by the Bryan strain of Rous sarcoma virus [RSV(-)]. The genome of this virus contains *src* but lacks the *env* gene. Virus particles produced by QCI-3 cells are thus not infectious due to the lack of the envelope glycoprotein^{11,14}. When QCI-3 cells are transfected with plasmids containing the *env* gene, *env* mRNA may be synthesized leading to production of infectious transforming RSV(-) particles. Stacey *et al.*¹⁴ have shown that the number of infectious virus produced is proportional to the amount of *env* mRNA available in the cell. This

Fig. 2 Structure of the retroviral clones used.

A, The structure of mRNAs from the *env* and rat preproinsulin II transcriptional units is shown. The region where transcripts initiating in the 5' LTR would terminate is shown to overlap with the region important for the initiation of transcription in the 3' LTR. B, The parental clone pBC8I is shown. The sequences shown were inserted between the *Sal*I and *Bam*HI sites of pBR322. The approximate location of the *env* gene intron is indicated. E refers to the sequence element noted by Schwartz *et al.*¹⁵ which lies between *env* and *src* in the Schmidt-Ruppin B strain used. (CAP, site of initiation of transcription; PA, polyadenylation signal; T = site of poly(A) addition and termination of viral mRNA). C, pBC8IΔ1 was constructed by *S*₁ exonuclease treatment of the *Sph*I site in the 5' LTR U3 region followed by insertion of a *Bgl*II linker DNA molecule. This results in a net insertion of 4 bp. D, pBC8IΔ2 was constructed by cleavage of the 5' LTR U3 region with *Pvu*I followed by *S*₁ exonuclease digestion and religation. A clone retaining the *Eco*RI site was then selected, and nucleotide sequence analysis revealed the deletion of 191 bp of the U3 region 5' to the *Eco*RI site. E, pBC8IΔ3 was created by deletion of the entire 5' LTR region and viral structural sequences, including element E¹⁵, located between the *Xho*I sites shown. The clone retains ≈1.5 kb of viral gene sequences in addition to the 3' LTR. F, pBC8I/PA contains a 237 bp *Bcl*I to *Bam*HI fragment from SV40 ligated into a *Bgl*II site in the *env* gene intronic region. This fragment has the SV40 late polyadenylation/termination signal inserted in the sense orientation relative to the *env* gene. G, pBC8IΔ4 was derived from pBC8IΔ3 by deletion of all viral structural gene sequences 5' to a *Mst*II site located ≈40 bp from the U3 region of the 3' LTR. H, pBC19I contains the previously described¹² LTR of the endogenous virus RAV-0 ligated to the rat preproinsulin II gene in the same fashion used for the exogenous virus LTR. It retains ≈130 bp of chicken DNA bounded by an *Eco*RI site. Clones pBC8IΔ3, pBC8IΔ4, and pBC19I were cloned between the pBR322 *Eco*RI and *Bam*HI sites in the opposite orientation to the other clones described relative to pBR322. pBR322 (---); retroviral sequences (—); preproinsulin sequences (=); S, *Sph*I; X, *Xho*I; R, *Eco*RI; H, *Hind*III; SL, *Sal*I; B, *Bgl*II; M, *Mst*II; Bm, *Bam*HI.



in turn is a reflection of the ability of the transfected plasmid to synthesize *env* mRNA.

The S_1 nuclease assay used to quantitate preproinsulin mRNA synthesis involves the use of a DNA probe uniquely end-labelled in an exon of the insulin gene¹³. As the probe extends through an intronic sequence of the insulin gene, annealing to correctly spliced preproinsulin mRNA synthesized in the QCI-3 cells followed by S_1 treatment results in the quantitative production of a band of characteristic size on a denaturing acrylamide gel. This S_1 assay does not distinguish between proinsulin transcripts originating in the 3' LTR and any readthrough transcripts which might initiate in the 5' LTR. As all the plasmid constructs contain the same preproinsulin transcription unit (Fig. 2), the steady-state levels of insulin mRNA as determined by the S_1 assay should be an accurate measure of the relative promoter activity of each clone tested.

Mutation of the upstream LTR

To determine whether the transcriptional activity of the upstream LTR affects that of the downstream LTR, we introduced mutations into the upstream LTR of plasmid pBC8I and measured the effect of these lesions both on *env* and preproinsulin gene transcription using the two independent assays. The different deletions introduced into pBC8I are outlined in Fig. 2.

In clone pBC8IΔ1 we have introduced a minor change into the U3 region of the upstream LTR by inserting a *Bgl*II linker DNA molecule into the *Sph*I site. This modification has been shown by DNA sequence analysis to result in the net insertion of four base pairs (bp). Clone pBC8IΔ2 has been shown by DNA sequence analysis to contain a deletion of the U3 region of the LTR extending up to the *Eco*RI site, within 57 bp upstream of the CAP site. In clone pBC8IΔ3 the entire upstream LTR and nearly all the viral structural gene sequences, including the *env* gene, are deleted leaving only the downstream LTR intact.

All modifications of the upstream LTR reduced *env* transcription relative to pBC8I, as determined by the *env* complementation assay (Table 1). In clone pBC8IΔ1 *env* synthesis was reduced only slightly. Clone pBC8IΔ2, although it retains part of the U3 region of the LTR including the 'TATA' box, shows only ≈1% the *env* gene activity of the undeleted clone. This supports the contention that the U3 region of the LTR contains sequences essential for promoting viral mRNA synthesis. As expected, clone pBC8IΔ3, which has a deleted *env* gene, produced no *env* activity. As may be seen in Fig. 3, mutation of the upstream LTR has the opposite effect on transcription of preproinsulin mRNA from the downstream LTR. Clone pBC8IΔ1 (lane 4) shows a small elevation in preproinsulin mRNA synthesis while clone pBC8IΔ2 (lane 3) and pBC8IΔ3 (lane 2) show clearly elevated levels compared to the parental clone pBC8I (lane 5). Clone pBC8IΔ3 shows consistently slightly lower levels of preproinsulin mRNA synthesis than clone pBC8IΔ2. The reason for this is unknown but may be due to the deletion of viral structural gene sequences in pBC8IΔ3 which have been suggested to affect transcription¹⁵. Alternatively this difference may be explained by the fact that, for technical reasons, pBC8IΔ3 is inserted into the plasmid vector in the opposite orientation compared to the other clones. Superinfection of QCI-3 cells with an avian leukosis virus has no effect on preproinsulin RNA transcription from transfected DNA templates (results not shown), demonstrating that envelope glycoprotein does not directly affect insulin mRNA synthesis.

As noted previously, the S_1 assay for preproinsulin expression would measure transcripts which initiate in the 3' LTR as well as any readthrough transcripts which initiate in the 5' LTR. Read through transcripts which fail to terminate using the LTR polyadenylation signal have been observed in avian cells⁹. The contribution of readthrough transcripts in this assay would result in an underestimate of the differences between intact and deleted LTR clones, so that the ≈5-fold difference in proinsulin mRNA transcription between pBC8I and pBC8IΔ2 may represent the minimum effect due to overlap interference.

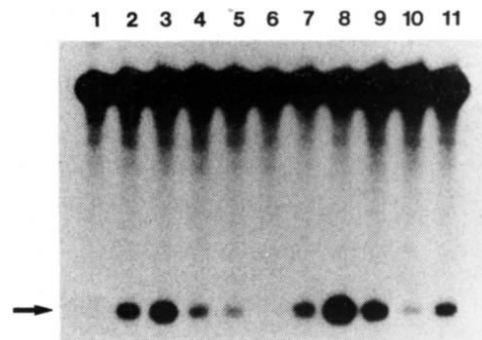


Fig. 3 Effect of 5' LTR promoted transcription on 3' LTR activity. Autoradiograph of S_1 assay acrylamide/urea gel. Lane 1, pR12/SV40-46; 2, pBC8IΔ3; 3, pBC8IΔ2; 4, pBC8IΔ1; 5, pBC8I; 6, pBC8; 7, 5 ng of rat insulinoma (RI) poly(A⁺)-RNA; 8, 25 ng of RI poly(A⁺) RNA; 9, pBC8I/PA; 10, pBC8I; 11, pBC8IΔ3. Lanes 1–5 and lanes 9–11 contain RNA samples derived from two independent transfection experiments. Relative *env* and proinsulin mRNA levels from transfected cells are given in Table 1.

Methods: QCI-3 cells are seeded at 9×10^6 cells per 100 mm dish 20 h before transfection. 5.8 μ g of clone pBC8I DNA or equimolar quantities of other plasmid DNAs were taken up in 2 ml of phosphate buffered saline (PBS) containing 1 mg ml⁻¹ of DEAE-dextran. The monolayer of QCI-3 cells was washed once with warm PBS, the DNA solution was added to the cells, and they were incubated at 37°C for 30 minutes with occasional gentle shaking. At this time, 25 ml of growth media was added to the cells and they were incubated for a further 2.5 h before refeeding with fresh growth media. Media samples for use in the *env* assay were taken at 72 h post-transfection. These were assayed for focus forming unit (FFU) production on chick embryo fibroblasts (CEF) as previously described^{11,14}. As the *env* gene used is of the B serotype, the viruses produced are infectious for CEFs but not for quail cells and thus no spread can occur in the transfected culture²⁹. After media sampling, RNA was isolated from the QCI-3 cells as described¹³. Total RNA yields were quantitated by UV absorbance, gel analysis, and dot blots using a *gag*-specific probe as the internal standard. Using 10 μ g of total RNA from each transfected culture, preproinsulin mRNA in each sample was quantitated by S_1 mapping^{13,30,31}. The probe used was a 750 bp fragment uniquely 3'-end-labelled at a *Bam*HI site in the coding region and extending to a *Hha*I site on the other side of the 499 bp intron in the preproinsulin II gene. Correctly spliced preproinsulin mRNA will anneal to the labelled anti-sense DNA strand, and nuclease S_1 will digest the unhybridized intronic sequences. A 170 nucleotide labelled fragment (indicated by arrow) is protected in proportion to the quantity of preproinsulin mRNA present in the reaction¹³.

Clone pBC8, which contains the same retroviral sequences as pBC8I but lacks the insulin gene, is negative in the preproinsulin RNA S_1 assay (Fig. 3, lane 6). If the preproinsulin gene is inserted in the inverted orientation relative to the LTR, no proinsulin mRNA is detectable (results not shown). Interestingly, clone pR12/SV40-46, in which the preproinsulin gene is under Simian virus 40 (SV40) early promoter control, also shows no detectable preproinsulin transcription in avian cells (Fig. 3, lane 1). This contrasts with the situation in monkey cells where this construction is quite active¹³. It is also of interest to note that the hybrid LTR U5 region-preproinsulin mRNA transcribed from the downstream LTR is able to efficiently program the synthesis and secretion of rat proinsulin (results not shown).

These results are consistent with the hypothesis that transcription from the upstream LTR in an integrated provirus inhibits transcription from the downstream LTR due to overlap interference. To prove this point directly, we have constructed clones in which transcription from the upstream LTR was terminated before reaching the downstream LTR.

Insertion of a transcriptional terminator

We have previously presented evidence that modification of the size of the *env* gene intron either by deletion or insertion of exogenous sequences has only limited effect on production of spliced *env* mRNA, given that the inserted sequences do not

Table 2 Relative *env* and proinsulin mRNA levels in the transfected cells

Lane	Clone	<i>env</i> activity		Insulin mRNA synthesis	
		FFU produced	Per cent	Peak area	Per cent
1	pBC8I	131,520	100	81	23
2	pBC8I/PA	260	0.2	346	100
5	pBC8IΔ4	0	0	308	100
6	pBC19I	0	0	46	15

Data are given as in Table 1. Lane nos refer to Fig. 4. Relative insulin mRNA levels were determined by peak area calculation using a DU-8 scanning spectrophotometer. mRNA levels are expressed as a percentage of clone pBC8I/PA (lanes 1 and 2) or pBC8IΔ4 (lanes 5 and 6).

interfere with transcription or contain splice sites¹¹. Thus, if we insert a transcriptional terminator into the *env* intron of clone pBC8I, reduced *env* production should be a measure of the premature transcriptional termination produced by this insertion. This is because any transcripts that avoid premature termination would be expected to be spliced in the normal manner, thereby eliminating sequences inserted into the intron and producing normal, functional *env* mRNA.

The transcriptional terminator we have chosen to use is derived from SV40 DNA. A 237 bp fragment containing a polyadenylation site was isolated following cleavage of SV40 DNA at the unique *Bcl*I and *Bam*HI sites. This fragment, which contains no splice sites used by SV40¹⁶, was inserted into the *env* gene intron in clone pBC8I such that the polyadenylation site used in SV40 late gene transcripts was in the sense orientation, thereby yielding clone pBC8I/PA (Fig. 2F). Transfection with DNA from clone BC8I/PA results in *env* production reduced by ≈99% relative to clone pBC8I (Table 1) but yields an increase in preproinsulin mRNA synthesis comparable to clone pBC8IΔ2 (Fig. 3). Thus, when transcription from the upstream LTR is prematurely terminated, the inhibition of the promoter activity of the downstream LTR is correspondingly relieved.

Endogenous retroviral LTR

The difference between the levels of transcription promoted by the 3' downstream LTR in the intact versus deleted clones is only about 5-fold in these experiments. This relatively small difference must be contrasted with the 30- to 100-fold increase in *c-myc* mRNA above basal levels found in the chicken bursal lymphomas¹⁷. However, small differences in the level of gene expression appear to have biologically significant consequences. We have previously presented evidence that the LTR promoter of the endogenous virus RAV-0 is ≈10-fold less efficient than the exogenous virus LTR in the *env* assay¹². RAV-0, which also produces 10-fold less mRNA in infected cells *in vivo*, does not cause tumours in chickens¹⁸. Evidence has been presented by ourselves and others^{12,18,19} that this lack of oncogenicity may be due to the lower efficiency of the RAV-0 LTR promoter.

To test the efficiency of the RAV-0 LTR for proinsulin transcription, two additional clones were constructed (Fig. 2, G, H). pBC8IΔ4 has a deletion of the entire viral transcriptional unit upstream of the 3' LTR, constructed by the removal of the remaining viral structural gene sequences in pBC8IΔ3. Only ≈40 bp of viral sequences remain in addition to the 3' LTR. Clone pBC19I contains the RAV-0 LTR in place of the exogenous Schmidt-Ruppin B LTR present in all other constructs, and is in the same orientation as clone pBC8IΔ4.

Data from a transfection experiment using these clones are shown in Fig. 4. In the *env* assay, pBC8I produces the greatest number of focus forming units while, as before, pBC8I/PA gives significantly less activity. pBC8IΔ4 and pBC19I yield no *env* activity as they lack the *env* gene. Quantitation of the preproinsulin mRNA assay (Table 2) shows that pBC8I/PA (lane 2)

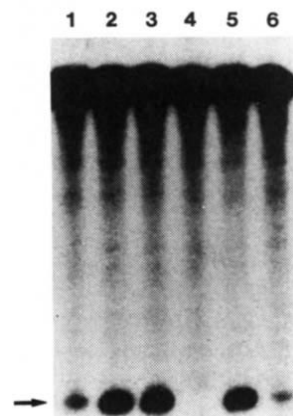


Fig. 4 Comparison to an endogenous retroviral LTR. Lane 1, pBC8I; 2, pBC8I/PA; 3, insulin mRNA positive control; 4, pBC8IΔ4; 5, pBC8IΔ4; 6, pBC19I. Relative *env* and proinsulin mRNA levels in transfected cells are given in Table 2.

Methods: Transfections were performed as outlined in Fig. 3 with the following minor modifications. Half as much DNA was transfected per culture (2.9 μg of pBC8I and equivalent molar amounts of other clones) and the cultures were treated for 2.5 h with 80 μM chloroquine in media as described by Luthman and Magnusson³², which results in an increase in transfected DNA uptake.

Nine μg of total RNA were analysed per lane.

produces the greatest amount of proinsulin transcripts, about 5-fold more than the pBC8I clone (lane 1). Transcription from pBC19I yields a low level of insulin mRNA (lane 6), ≈7-fold less than pBC8IΔ4 (lane 5). Thus, the relatively small difference in insulin transcription between the RAV-0 LTR and the exogenous LTR in the deleted clones, which appears sufficient to distinguish a non-oncogenic from an oncogenic LTR promoter, is comparable to the difference caused by transcriptional interference in the downstream LTR of an exogenous retrovirus.

Implications for ALV leukaemogenesis

We have presented evidence that viral mRNA transcription extending into the downstream LTR of a provirus-like construct restricts the ability of this LTR to function as an efficient promoter. The inhibition, which we term transcriptional overlap interference, is removed if the upstream LTR is damaged or deleted or if a transcriptional termination signal is inserted between the LTRs. The increased ability of the downstream LTR of an integrated provirus to act as a promoter following deletion of the upstream LTR provides an explanation for the observation that only deleted proviruses are observed inserted upstream and in the same orientation to *c-myc* in lymphomas of chickens⁵⁻⁷.

Alternative configurations of the integrated provirus relative to *c-myc* have been described by Payne *et al.*²⁰. One bursal lymphoma contained a provirus downstream of *c-myc* in the same orientation, while others had the proviral DNA upstream of *c-myc* in the opposite orientation. In the former case, the provirus was deleted, leaving only the 3'-LTR and adjacent viral DNA. In addition, at least 15 kb of cellular DNA on the 3' side of *c-myc* was also lost. In the latter cases, deletions of the provirus are also observed. These deletions are, however, internal in most, though not all, cases^{6,20}. These two alternative configurations do not allow the direct use of the LTR as the promoter for *c-myc* expression, as predicted by the promoter insertion model. Thus, deletion of LTR sequences would not be required to relieve overlap interference in these cases.

The pathology of lymphoid leukaemia in chickens sheds additional light on how neoplastic transformation may proceed²¹. Following infection of newborn chicks by ALV, the earliest histologic evidence of the preneoplastic state is the appearance of transformed follicles in the bursa. A small proportion of these follicles (≈2%) give rise to nodules which latter progress to a disseminated lymphoid leukaemia. This multi-step progression to lymphoid tumours may represent discrete events at the

molecular level. Our data are consistent with the hypothesis that malignant transformation may begin by the rare random integration of an ALV provirus adjacent to *c-myc*, which would interrupt the *c-myc* locus. This first event would result in the disruption of normal cellular regulation of the *c-myc* allele, followed by increased expression of *c-myc* promoted by the 3' LTR of the integrated provirus. A second random event, deletion of the LTR, would cause an additional increase in *c-myc* transcription. It has previously been observed that integrated proviruses may spontaneously delete part of the viral sequence, including an LTR, at a low frequency²². These two discrete events may be necessary to allow *c-myc* transcription promoted by the remaining LTR to increase to levels sufficient to cause malignant transformation. Subsequent events, such as the postulated activation of a second oncogene²³, may also be required to produce leukemia.

The control of gene expression

The frequency with which the phenomenon of transcriptional overlap interference occurs remains to be determined. A process analogous to overlap interference has been reported in prokaryotes where it has been shown that the upstream P_L promoter of prophage λ inhibits the initiation of transcription at downstream *gal* promoters²⁴. Interference between overlapping transcriptional units has also been observed in other prokaryotic operons^{25,26}. Therefore, the phenomenon of overlap interfer-

ence appears not to be unique to the avian provirus but could represent a novel mechanism for the control of gene expression. Transcriptional overlap interference could be used to coordinately regulate two adjacent, overlapping transcriptional units by controlling only the upstream promoter directly. The downstream promoter would become progressively more active as the upstream promoter was repressed and less active as the upstream promoter was induced. A possible example of this phenomenon has been observed in yeast²⁷. The 5' gene in this case is the repressible acid phosphatase gene *PHO5* while the 3' gene is the acid phosphatase gene *PHO3*. These genes are closely linked in yeast and were cloned together on a single 8 kb DNA fragment. *PHO3* is expressed in yeast growing in media containing phosphate while *PHO5* is repressed. If phosphate is removed *PHO5* is induced and *PHO3* is no longer detectable²⁷. It has now been demonstrated (R. Kramer and K. Bostian, personal communication) that if the upstream *PHO5* gene promoter is deleted, the *PHO3* gene continues to be transcribed in the absence of phosphate. These observations are consistent with the use of transcriptional overlap interference as a mechanism in the coordinate control of differential gene expression.

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LETTERS TO NATURE

A new kind of natural radioactivity

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From a systematic study of the properties of nuclei heavier than lead, we have concluded that, in one or two cases, radioactive decay by emission of a particle heavier than the α -particle might be observable in competition with the latter. We have observed such decay in ^{223}Ra which occurs in the natural radioactive series emanating from ^{235}U . Two 'by-pass' modes seem likely: emission of ^{14}C , to ^{209}Pb , with a Q -value of 32 MeV; and of ^{12}C , to ^{211}Pb , with a Q -value of 28 MeV. (Emissions of ^{13}C and ^{15}C from the same nucleus have *a priori* emission rates similar to that of ^{12}C , but the emission of α -like nuclei seems more likely.) In this study we have used a solid-state counter telescope to identify the charge of the particle emitted, and, for greater convenience, a source of ^{227}Ac (half life 21 yr), with which the lower members of the series, including ^{223}Ra , are in secular equilibrium. We have observed particles identifiable as carbon ions and, from their energy and emission rate, they are ^{14}C rather than other carbon isotopes. The branching ratio for emission of ^{14}C nuclei relative to α -particles from ^{223}Ra is $8.5 \pm 2.5 \times 10^{-10}$, corresponding to a reduced width (preformation probability) smaller by a factor of $\sim 10^5$ to 10^6 .

The obvious problem in the detection of rare modes of decay is to distinguish between these modes and the multiple pile-up of α -particle pulses. Using techniques familiar in nuclear experimentation, we were able to reject events due to pile-up provided that individual α -particles in the event were separated in time by over ~ 100 ns. Even so, with a detected α -particle rate of $\sim 4,000 \text{ s}^{-1}$ we observed, over a counting period of several months, events extending out as far as quadruple pile-up of total energy up to 28 MeV. However, the ratio of ionization in the thin (ΔE) counter of the telescope to that in the thick ($E - \Delta E$) counter for a carbon ion, is quite different from the same ratio arising from α -particle pile-ups of the same or nearly the same total energy, so that all the α -events including multiple pile-up, are clearly segregated from carbon events in a $\Delta E, E$ plot. In any case, the total number of quadruple α -pile-ups to within 5 MeV of our detected events was small; see Fig. 1.

Another problem, which resulted in our doubting the energy measurements of, and consequently rejecting, a run of several months, is that of radiation damage occurring in the semiconductor detectors exposed to a high counting rate over a long period of time. As the ionization density for α -particles is at its highest very close to the end of the range, the $E - \Delta E$ counter responds to damage more quickly than the ΔE counter. Because the α -particles and the carbon ions at the energies of the experiment have much the same range, one cannot reduce the $E - \Delta E$ counter in thickness until it no longer stops the α -particle, but

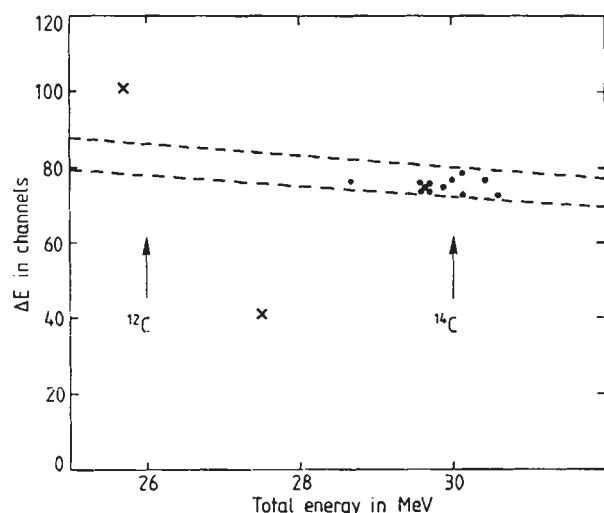


Fig. 1 Contents of the two-dimensional array ΔE versus E_{total} after a run of 189 days. The dotted line indicates the allowed region for carbon ions and the arrows indicate the total energies expected for ^{12}C and ^{14}C emissions in the decay of ^{223}Ra . The lower of the two crosses represents a quadruple pile-up. Below the total energy displayed, large numbers of triple and double α -pile-ups were recorded. Single α -events (and, in part, even double α -pile-ups) were biased out on the analogue side to avoid deadtime problems on the digital side. The upper cross is an event which was recorded during a thunderstorm which affected the mains badly. A run of 194 days was made before this one, yielding 8 events and, in addition, a run of approximately half a year was performed to investigate possible cosmic ray-induced events. Channel 77 in $\Delta E = 6.7$ MeV, which is exactly as expected for 30 MeV ^{14}C . Detector characteristics: The dead layer of the ΔE detector (200 mm² active area, 8.2 μm sensitive thickness) was determined to lie between 0.3 and 0.8 μm . In addition a protective layer of gold of thickness 20 $\mu\text{g cm}^{-2}$ was evaporated on the source and 15 $\mu\text{g cm}^{-2}$ carbon film inserted between the source and the ΔE detector. An extra 30–40 $\mu\text{g cm}^{-2}$ of gold is present on the E-detector (300 mm² active area). This gives a total of 150–250 $\mu\text{g cm}^{-2}$ of effective dead layer (Si equivalent) and an energy loss of ^{14}C ions of 0.5–0.8 MeV. The source of strength 3.3 μCi gave a counting rate of $\approx 4,000 \text{ s}^{-1}$, corresponding to an effective solid angle of detection of $\approx 1/3$ sr.

still stops the higher charged particle (a technique used in fission measurements, using gas counters, in order to enhance the fission pulses relative to α -pulses and so overcome the effects of pile-up). After having experienced this effect with a thick detector which had been much used previously, the problem was overcome by using new counters and monitoring closely the single α -pulses in both detectors. While the events detected in the first run were clearly due to carbon ions, the energy measurements were uncertain to ~ 2.5 MeV, but in the second run the energy measurements were reliable and close to the expected value for ^{14}C emission, that is, 30 MeV, after allowing for the recoil energy of the daughter nucleus (see Fig. 1). The runs were done automatically using on-line LSI-11/Camac-based two-dimensional storage techniques. To mitigate against hardware failure, the results were read out and up-dated every 12 h. In the event of an error occurring during the 12-h period, the computer was programmed to reject that 12-h run and to carry on from the previously stored data.

Before discussing further the energy measurements, the accompanying table is of interest. Of all the exotic emissions possible from the radioactive chain we are using, we have listed those which are most likely to be observed and have given the ratio of their Gamow factors to the appropriate Gamow factors for α -emission for three values of the parameter r_0 in the expression for channel radius, $r = r_0(A^{1/3} + a^{1/3})$, where a represents the mass number of the emitted particle and A that of the daughter nucleus (see Table 1). It is immediately evident that emission of ^{14}C from ^{223}Ra virtually selects itself as the

Table 1 Possible strange decay modes in the ^{235}U natural ($4n+3$) radioactive series and their relative probabilities for observation

Decay	Q-value (MeV)	Gamow factor for decay/Gamow factor for α -emission		
		$r_0 = 1.15f$	$r_0 = 1.2f$	$r_0 = 1.25f$
$^{227}\text{Th} \rightarrow ^{19}\text{O} + ^{208}\text{Pb}$	44.15	4.5×10^{-11}	2.2×10^{-9}	9.7×10^{-8}
$^{227}\text{Th} \rightarrow ^{28}\text{Mg} + ^{199}\text{Pt}$	68.25	8×10^{-17}	3.5×10^{-13}	1.0×10^{-10}
$^{227}\text{Th} \rightarrow ^{14}\text{C} + ^{213}\text{Po}$	29.4	4.3×10^{-12}	7.7×10^{-11}	1.2×10^{-9}
$^{227}\text{Th} \rightarrow ^{18}\text{O} + ^{209}\text{Pb}$	44.2	2.2×10^{-10}	1.1×10^{-8}	4.1×10^{-7}
$^{227}\text{Ac} \rightarrow ^{13}\text{N} + ^{212}\text{Pb}$	33.3	2.8×10^{-10}	5.6×10^{-9}	1.2×10^{-7}
$^{223}\text{Ra} \rightarrow ^{13}\text{C} + ^{208}\text{Pb}$	29.1	5.3×10^{-12}	1.0×10^{-10}	1.8×10^{-9}
$^{223}\text{Ra} \rightarrow ^{14}\text{C} + ^{209}\text{Pb}$	31.8	1.2×10^{-5}	1.7×10^{-4}	2.2×10^{-3}
$^{223}\text{Ra} \rightarrow ^{13}\text{C} + ^{210}\text{Pb}$	28.8	1.1×10^{-10}	1.7×10^{-9}	2.1×10^{-8}
$^{223}\text{Ra} \rightarrow ^{12}\text{C} + ^{211}\text{Pb}$	27.7	6.7×10^{-12}	8.5×10^{-11}	1.0×10^{-9}
$^{219}\text{Rn} \rightarrow ^{14}\text{C} + ^{205}\text{Hg}$	28.03	1.5×10^{-16}	2.7×10^{-15}	4.1×10^{-14}
Normalizing Gamow factor for α -emission				
$^{227}\text{Th} \rightarrow \alpha + ^{223}\text{Ra}$	6.15	4.9×10^{-6}	2.2×10^{-5}	9.8×10^{-5}
$^{227}\text{Ac} \rightarrow \alpha + ^{223}\text{Fr}$	5.04	1.3×10^{-11}	6.2×10^{-11}	2.8×10^{-10}
$^{223}\text{Ra} \rightarrow \alpha + ^{219}\text{Rn}$	5.98	6×10^{-6}	2.7×10^{-5}	1.2×10^{-4}
$^{219}\text{Rn} \rightarrow \alpha + ^{215}\text{Po}$	6.94	5.5×10^{-1}	2.3	9.3

The full Q-values were used in the computations. The major α -modes are known to lead to ground states and/or states nearby (that is, within <300 keV above ground state); l -values can be neglected in the strange decay modes; the considered α -decays for normalization are all leading either to the ground state (or a state nearby) with an $l=0$ or $l=2$ branch, the branching ratio being sufficiently strong for the order-of-magnitude estimates presented.

explanation of our data; emission of ^{12}C , ^{13}C or ^{15}C from the same parent are equally likely (*a priori*) to within a factor of 10, but considerably less so than ^{14}C emission. Since sequences of three α -particles often occur in radioactive chains, one may reasonably expect the preformation probabilities for ^8Be , ^{12}C and ^{16}O to be considerably higher than for other configurations; but there seems to be no good reason to assume similar enhancements for ^{13}C and ^{15}C relative to ^{14}C . Considering the data in Table 1, the most likely emission observed is ^{14}C , with ^{12}C (and a more favourable preformation probability) the next most likely. Therefore, we have given the location of the ^{12}C group in Fig. 1.

If we now introduce our measured ratio of $8.5 \pm 2.5 \times 10^{-10}$ carbon nuclei per α -particle and compare it with the Gamow ratio of 1×10^{-5} to 2×10^{-3} (see Table 1) for ^{14}C , 7×10^{-12} to 1×10^{-9} for ^{12}C , 1×10^{-10} to 2×10^{-8} for ^{13}C , and 5×10^{-12} to 2×10^{-9} for ^{15}C , we conclude that the preformation probabilities for ^{12}C , ^{13}C and ^{15}C need to lie somewhere in the range 0.1–10 times that for the α -particle; we consider this to be preposterous for ^{13}C and ^{15}C and unacceptable for ^{12}C . Thus, we conclude that the emissions are of ^{14}C from ^{223}Ra with a preformation probability in the range of 7×10^{-5} – 4×10^{-7} times that for α -emission from the same nucleus. These considerations relegate the energy calibration to a somewhat minor role, although important to us as it is a measure of confidence in what we are doing.

The energy calibration was made using ^{241}Am α -particles of 5.48 MeV, and extrapolating up. This may seem rather risky when it is realized that the source was covered by thin gold and carbon foils, to prevent source material sputtering onto the ΔE counter due to daughter recoil on α -decay, and in addition the detectors themselves have unknown dead layers. However, serendipity occasionally comes to the aid of the experimenter; from tables of energy losses of charged particles, it turns out that ^{241}Am α -particles and ~ 31 -MeV ^{14}C ions have almost the same ratio of dE/dx to E thus the fractional energy loss for each particle is the same. Hence the pulse outputs should scale as the total energies independently of the unknown energy losses, provided they are fairly small (extrapolation from energy to energy even for different types of particle is well established for Si semiconductor detectors). As the unknown energy loss is estimated (see Fig. 1 legend) to be of the order of ≤ 1 MeV for carbon ions of these energies, a linear scaling through the origin and the 31-MeV point will produce very little error in the neighbourhood of 30 MeV. It is, therefore, highly gratifying to us that our extrapolated calibration assigns to the closely

grouped events observed almost exactly the energy required for ^{14}C emission from ^{223}Ra .

Finally, we feel that similar by-pass decays must occur in other nuclides in this region, chiefly to the doubly magic nucleus ^{208}Pb and its near isotopes in order to make use of the extra stability of the daughter nucleus. As the neutron excess increases rapidly with Z , the parents will tend to be more neutron-deficient than nuclides in the naturally occurring sequences which are on the neutron-rich side of the valley of stability. The likely candidates for by-pass decay will probably need to be man-made (or arise in series in which the ultimate parent is man-made) rather than occur naturally.

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Power spectrum of long-period solar oscillations and 160-min pulsations during 1974–82

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We report here the results of solar oscillation observations carried out between 1974 and 1982 using the Crimean solar magnetograph. We have eliminated all peaks of telluric origin from the mean power spectrum; of the remaining 32 dominant ($>2\sigma$) peaks, 19 are of solar origin and 13 are partly blended with the atmospheric peaks. We found that 10 solar peaks are in very good agreement with the modes g_{10} – g_{20} of degree $l = 4$ calculated by Christensen-Dalsgaard *et al.*¹ for the solar model C with the envelope enriched in heavy elements by accretion. Deviations of the observed long-period oscillations from a linearity can be appreciable, leading to the appearance of combination frequencies. The dominant 160-min peak seems to be excited by a strong 'double' resonance of $2g_8$ mode of model C with a combination frequency, and two other combination frequencies of modes $1g_1$ and $2f$ including the unstable mode $l = 1$. As model C is also in good agreement with the neutrino experiment, we suggest that this could be considered as the most appropriate model of the Sun. In contrast to previous detailed studies of solar pulsation with the particular 160.01-min period^{2,3}, the emphasis here is on the search for other possible long-period solar oscillations of low degree. These oscillations are thought to be bound to the regions beneath the convection zone and penetrate to the Sun's core which makes them a powerful tool for analysing the solar internal structure and the evolution of the Sun.

The Crimean solar magnetograph with a special Doppler shift modification⁴ is being used continuously to measure the wavelength difference between two profiles of the same nonmagnetic spectral line $\lambda 5,124 \text{ \AA}$ —one from the central circular area ($0.66R_\odot$) of the solar disk, and the other from the remaining annular portion of the solar disk⁵. The observations described here were made in 1974–82 during 473 days spanning 2,719 h (for data processing see ref. 5). The double solar magnetograph was used to discriminate real solar peaks in power spectrum from false peaks produced mainly by the Earth's atmosphere (such as fluctuations of transparency and differential extinction). Simultaneous observations over 7 yr of the telluric $\text{O}_2\lambda 6,879 \text{ \AA}$ line (1975–82, 905 h in total) were carried out and reduced in a similar way.

The power spectra of the mean 5-min-interval residuals (centre minus limb data) were calculated for both spectral lines in three ways: (1) independent mean power spectra were obtained for each annual data set; (2) the total 1974–82 data

Table 1 Real solar peaks

No. of peak	Dominant peaks of solar spectrum (1974–82) $T(\text{min})$	Identification with the model C	
		$l = 4$ (O–C, min)	$l g_n - l' g_i$
1	200.5		$2g_2-2g_4$ (200.6); $2g_1-4g_5$ (200.5); atm?
2	200.0 S	$g_{20} = 199.9$ (0.1)	$4g_{20}$
3	196.3		$1g_2-4g_{21}$ (196.5)
4	192.6*		$2g_3-4g_{14}$ (192.3); atm?
5	192.0*S	$g_{19} = 191.8$ (0.2)	$4g_{19}$
6	188.5*S		$1g_1-2g_5$ (188.3)
7	184.7		$3g_4-4g_{12}$ (184.9)
8	183.5	$g_{18} = 183.6$ (–0.1)	$4g_{18}$
9	180.2 S		$2g_3-4g_{15}$ (180.1)
10	175.6 S	$g_{17} = 175.5$ (0.1)	$4g_{17}$
11	173.9*		$1g_1-3g_8$ (173.7)
12	171.0		$3g_4-4g_{13}$ (170.8); atm?
13	168.3		$3g_2-4g_7$ (168.4); atm?
14	166.9	$g_{16} = 167.2$ (–0.3)	$4g_{16}$
15	160.0 S		$2g_8$ (160.80); $1g_1-2f$ (160.83); $2g_1-4g_6$ (160.24); $3g_4-4g_{14}$ (160.21)
16	159.3	$g_{15} = 158.8$ (0.4)	$4g_{15}$
17	157.1		$1g_1-2g_6$; (157.2); atm?
18	153.1 S		$1g_1-4g_{12}$ (152.85); atm?
19	151.8*		$3g_4-4g_{15}$ (151.6); atm?
20	150.0*	$g_{14} = 150.3$ (–0.3)	$4g_{14}$
21	148.4		$3g_3-4g_{11}$ (148.1)
22	144.0		$3g_1-4g_5$ (144.2); atm?
23	143.2		$1g_1-4g_{13}$ (143.0); atm?
24	142.0	$g_{13} = 142.1$ (–0.1)	$4g_{13}$; $2g_3-4g_{21}$ (141.7)
25	139.4		$1g_1-2g_7$ (139.6); $2g_2-4g_{13}$ (139.2); $3g_4-4g_{17}$ (139.0)
26	134.3*S	$g_{12} = 133.6$ (0.7)	$4g_{12}$; atm?
27	130.1*S		$3g_3-4g_{13}$ (129.8)
28	129.3		$1g_1-4g_{15}$ (129.3)
29	122.3*S		$3g_1-4g_6$ (122.1); $2g_1-4g_8$ (121.9); atm?
30	120.4 S		$2g_1-1g_2$ (120.03); $3g_2-4g_{10}$ (120.45); $3g_8$ (120.90)
31	119.5		$1g_1-3g_{13}$ (119.7); atm?
32	116.4 S	$g_{10} = 116.6$ (–0.2)	$4g_{10}$; atm?

* Peaks not denoted on Fig. 1 (amplitudes ones which are dominant ($>2\sigma$) in the string of 8-year data (1974–81)). Separate peaks 153.3 and 152.9 min have been combined in one peak 153.1 min in accordance with resolution adopted. The same was done with the peaks 170.8 and 171.2 min.

set was considered and the coherent spectrum calculated (with a resolution of 1 nHz); (3) nine annual power spectra were normalized and subsequently averaged. The later spectra (with an effective resolution of $\sim 0.07 \mu\text{Hz}$) in the period range 110–210 min is shown in Fig. 1. Besides the apparent presence of a dominant peak at 160.0 min, there are several other peaks of comparable and lower amplitudes.

To discriminate the real solar peaks of this spectrum we used the similar spectrum of the telluric $\lambda 6,879 \text{ \AA}$ oxygen line. However, before comparing the solar and atmospheric power spectra we have considered the meaning of the 'coincidence of peaks'. A careful study of the positions of previously selected 93 dominant peaks in the solar and telluric power spectra, based mainly on the comparison of above mentioned three types of spectrum, indicated that the real error in the period's value is $|\Delta T| \leq 0.5 \text{ min}$ for periods $\approx 160 \text{ min}$. We were able to assign 37 peaks to the telluric power spectrum and they were thus excluded. The 17 peaks observed only at Stanford (1977–80 data) or only at the South Pole were also excluded. Seven peaks that were observed but were not identified with theory¹ were not considered. Of the remaining 32 peaks (listed in Table 1),

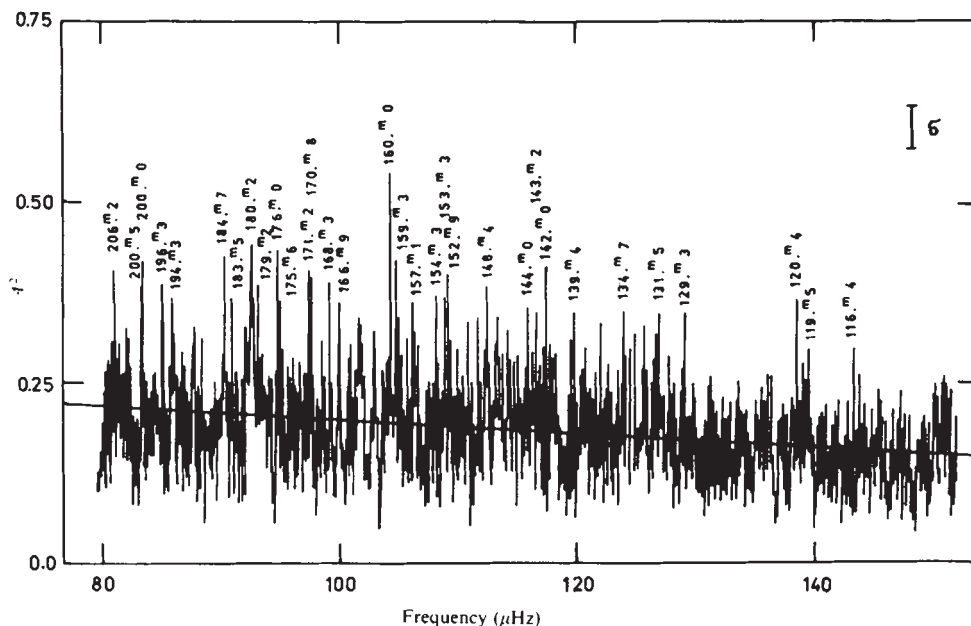


Fig. 1 Power spectrum of solar oscillation, 1974-82. The numbers indicate periods (in min) of dominant peaks, exceeding the 2σ confidence level. Straight line represents the mean level, vertical bar corresponds to 1σ deviation from the mean level.

13 are partly blended with the atmospheric peaks (notified 'atm?') and 19 can be considered as of real solar origin. All these 32 peaks (periods, in minutes) are given in Table 1. Those 12 peaks which coincide with those observed at Stanford (during 1977-80) are denoted (by S). The data obtained at the South Pole are not used here because they are rather scarce and add no essential features either to Table 1 or the echelle-diagram (except one point with the period 204.0 min; the other four points coincide well with our points). Table 1 contains 23 peaks from the 1974-82 data (see Fig. 1) and nine peaks from the 1974-81 data which are dominant for all this time interval.

To estimate the noise level of the power spectrum shown in Fig. 1, the mean of amplitudes and r.m.s. deviation σ_0 associated with it were computed (zero-order approximation). Then all the peaks exceeding the $3\sigma_0$ level were excluded and the new mean amplitude and corresponding r.m.s. (σ_1) were recalculated. Then, all peaks exceeding the $2\sigma_1$ level were excluded, and the final mean amplitude and final r.m.s. noise $\sigma \approx 0.054$ ($\text{m}^2 \text{s}^{-2}$) were computed (see Fig. 1 where 32 peaks with amplitudes $A^2 \geq 2\sigma$ are denoted), 23 of these peaks which can be identified are included in Table 1. These 23 peaks are also present in the 1974-81 data which show nine more significant and identified peaks. (Note that the amplitude of 160-min peak is $\approx 6\sigma$).

Having tabulated the periods and considered them as eigenmodes of solar model oscillations, one can immediately find close correspondence of these observed periods with those calculated by Christensen-Dalsgaard *et al.*¹ for their model C of the Sun for degree $l=4$ and orders ranging from g_{10} to g_{20} . The values $4g_n$ calculated in (ref. 1) and taken from their Table 3 are presented in Table 1, as well as on the echelle-diagram (see Fig. 2, where the dashed straight lines connect theoretical values).

There is satisfactory agreement between theoretical $4g_n$ values and a string of the observed ones: the mean O-C = 0.05 min for all 10 values. The probability of chance coincidence, within a tolerance ± 0.5 min, here $\approx (\frac{1}{2})^{10} \sim 10^{-3}$. The Monte Carlo method, for the determination of probability of the simultaneous chance coincidence, gives the value $\sim 3 \times 10^{-4}$ for 10 g -modes periods (the tolerance window being $\pm 0.33 \mu\text{Hz}$). We have failed to find a similar significant correspondence for $l=1$, 2, 3 degrees of C as well as for models A and B of ref. 1.

The mean difference in the observed periods $\Delta\bar{T}$ of successive modes g_n listed in Table 1 is $\Delta\bar{T}_{\text{obs}} = 8.36 \pm 0.60$ min. According

to the well known asymptotic approximation formula

$$T_{nl} = K \left(n + \frac{l}{2} - \frac{1}{4} \right) (l(l+1))^{1/2} \quad (1)$$

and $\Delta\bar{T}_{\text{obs}}$ we have $K_{\text{obs}} = 37.4 \pm 2.7$ min ($l=4$). On the other hand, Christensen-Dalsgaard *et al.*¹ give $K_{\text{theor}} = 37.6$ min for model C, which is in good agreement with observations, and $K_{\text{theor}} = 36.2$ min for the standard A-model. If one now compares these values with the $K = 38.6$ found recently by Delache and HScherrer⁶ from a very limited set of data (one season during 1979) and for the range of periods mainly $P \geq 200$ min, one comes to the conclusion that their value (38.6 min) is appreciably nearer to our value for the C-model than to K for the standard A-model. The same is true for observations of van der Raay⁷ producing the value $K \approx 41.2$ min. As all these authors deal with $l=1$ or 2 degrees and use a standard model we can conclude that C-model values fit the observed values better than do those of the standard A-model. (However, the disagreement between Delache and Sherrer's K -value and that of van der Raay⁷ is quite large, and is ≥ 10 min for periods ≥ 160 min. Difficulties with the standard model and the necessity of using the lower abundance Z of heavy elements for p -modes ($l < 20$) has recently been pointed out by Rhodes *et al.*⁸, see also ref. 9.)

The following evidence favours a deviation of the observed oscillations from linearity. (1) The observed dependence of the amplitude of oscillations on the phase of solar rotation¹⁰ supports the fact that we also observe asymmetric dipole-like ($l=1$) oscillations contributing to the power spectrum. (2) The recent investigation of Kosovichev and Severny¹¹ showed that instability really takes place for the $l=1$ degree in the C-model (for g_1 and g_2 -modes; compare with the similar conclusions of Saio¹²). (3) The amplitudes of g -modes increase towards the centre (the core) of the Sun. (4) If one compares the value of harmonic mean of the observed amplitude $\bar{a}_h = 0.67$ with the mean amplitude of bimodal terms $\bar{a}_b = (a_i a_j)^{1/2} = 0.71$ formed for the dominant peaks, one finds them quite comparable. (S. M. Ruitov, personal communication).

Therefore, because the oscillations are nonlinear, the combination frequencies $\omega_i \pm \omega_k$ can occur (see ref. 13). The corresponding combination frequencies for different peaks were also calculated and are presented in Table 1. The corresponding periods are in fairly good agreement with the observed values.

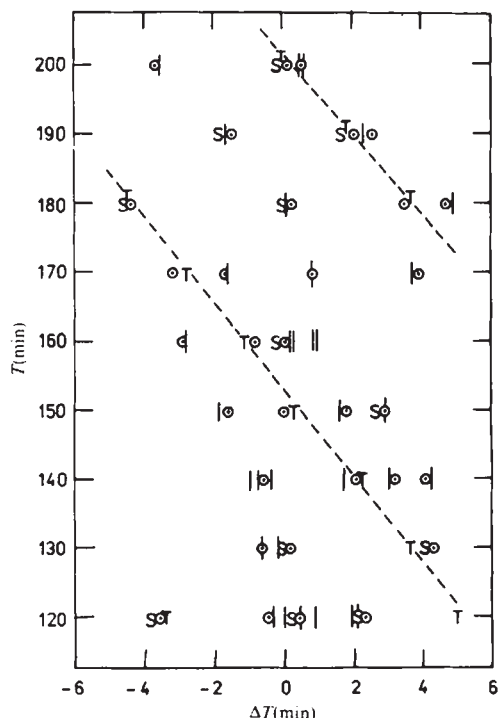


Fig. 2 Echelle-diagram of the positions of all peaks with amplitude $\geq 2\sigma$ for periods between 110 and 210 min constructed with the increment $\Delta T = 10$ min. ○, Solar periods; S, peaks in the Stanford spectrum coinciding with ours; T, periods of theoretical $4 g_n$ -modes for model C and $l = 4$; 1, combination periods (see Table 1). The dashed lines show the sequences of the theoretical $4 g_n$ -modes.

In conclusion, let us consider 160.0-min oscillation (as well as the 120.4 and 139.4-min peaks). Besides that, at $l = 2$ the g_8 mode does exist for which the calculated value (see Table 3 in ref. 1)

$$T(2g_8) = 160.80 \text{ min} \quad (2)$$

and the f - and g_1 -modes (the latter can be secularly unstable according to Saio¹²) form the combination mode with the period

$$[T^{-1}(2f) - T^{-1}(1g_1)]^{-1} = 160.83 \text{ min} \quad (3)$$

which is almost in resonance with $T(2g_8)$ -mode, as $\Delta T_1 = 0.03$ min. Note that $1g_1$ and $2f$ are the lowest modes of model C. There also exists another resonance near the 160-min-period produced from the combination modes

$$\begin{aligned} [T^{-1}(2g_1) - T^{-1}(4g_6)]^{-1} &= 160.24 \text{ min} \\ [T^{-1}(3g_4) - T^{-1}(4g_{14})]^{-1} &= 160.21 \text{ min} \end{aligned} \quad (4)$$

which also have $\Delta T_2 = 0.03$ min, so that there is a 'higher'-order resonance with $\Delta T_3 = \Delta T_2 - \Delta T_1 = 0$ or a 'resonance of two resonances' with $\Delta T = 0.03$ min. If for model C the mode $l = 1$ is unstable (see refs 11, 12) the presence of a resonance mode (3) (and possibly $l = 2$ resonance mode (4)) can lead to a secularly unstable mode near 160.0 min. The possibility of resonance excitation of the 160-min mode was also suggested by Vandakurov¹⁴, but this author had neither proper observational data nor theoretical modes, so that the actual condition for resonance differs from those supposed in ref. 14. The problem of resonance in other cases is now being considered separately, but it is obvious that only a very slight adjustment to model C is needed to make resonance appear precisely at the observed frequency 160.01 min. (For the possible importance of resonance coupling see also ref. 15, and ref. 16 for the instability connected with it).

A similar situation presents itself at $T = 120.4$ min (confirmed by the two Stanford peaks having the mean period 120.1 min). Here we have the secular unstable modes $l = 1$ in two combina-

tion frequencies:

$$\begin{aligned} [T^{-1}(2g_1) - T^{-1}(1g_2)]^{-1} &= 120.03 \text{ min} \\ [T^{-1}(3g_2) - T^{-1}(4g_{10})]^{-1} &= 120.45 \text{ min} \end{aligned} \quad (5)$$

also forming a very close resonance with $\Delta T_1 = 0.42$ min. There also exists a normal mode $l = 3$, g_8 with the period 120.90 min producing the difference $\Delta T_2 = 0.45$ min, so that we again have the condition $\Delta T_3 = \Delta T_2 - \Delta T_1 = 0.03$ min indicating the very close resonance conditions. We think the precise resonance at 120.4 min may mean a very slight change in model C.

We have tried to find out the near-resonance conditions for the standard A-model but have failed so far. Therefore, we have come to the conclusion that model C, which is also in much better agreement with the neutrino experiment than the other two (A and B) models of ref. 1, is a better model of the Sun, and thus the enrichment of solar atmosphere by heavy elements could be connected with the accretion process.

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Early Solar System aqueous activity: Sr isotope evidence from the Orgueil CI meteorite

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The CI meteorites are rare but important objects because they may represent our best sample of chemically unfractionated Solar System material (see, for example, ref. 1). Despite the fact that these meteorites apparently retain their original chemical composition, they clearly contain secondary mineral phases, some at least believed to have been produced through the action of liquid water on the parent body^{2–6}. The timing of this event, however, was unknown. In an attempt to solve this problem, we have measured the Sr isotopic composition and ⁸⁷Rb/⁸⁶Sr in carbonates and sulphate separated from the Orgueil meteorite. Both of these phases probably precipitated from aqueous solution. Our first results, reported here, show that carbonate deposition occurred contemporaneously with parent body formation or shortly after it, probably within 100 Myr. On the other hand, at least some of the calcium sulphate seems to have been formed recently.

In the CI meteorites, the Rb/Sr ratio is high, ~ 0.3 (ref. 7). Thus, in low-Rb phases which contain Sr, the Sr isotopic composition should be a sensitive indicator of the time of formation,

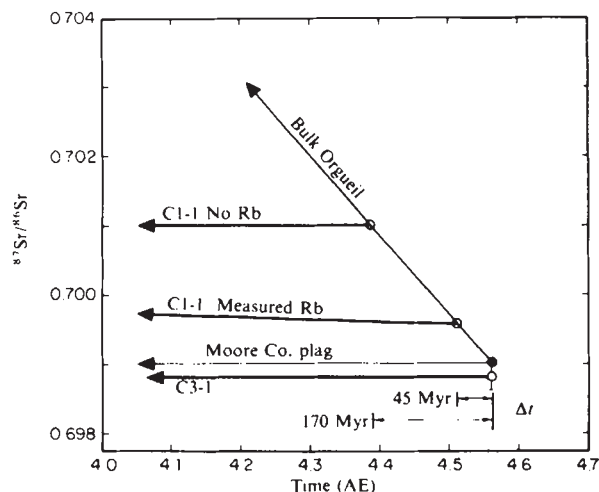


Fig. 1 An illustration of the Sr isotope method for determining the time of formation of low-Rb phases such as carbonates in the CI meteorites, using data for C1-1 (Table 1) as an example. Evolution lines are shown for the bulk meteorite ($^{87}\text{Rb}/^{86}\text{Sr} \sim 0.8$), the Moore Co. plagioclase, and sample C1-1 assuming as extreme cases that (1) the sample has no indigenous Rb and (2) the measured Rb is the true value. For purposes of calculation, no blank correction was made to the Rb data for case (2), so that $^{87}\text{Rb}/^{86}\text{Sr} = 0.022$ rather than 0.013 as reported in Table 1. This results in a lower limit for Δt (the time between 4.56 AE and the isolation of C1-1 from bulk Orgueil Sr). Also shown for comparison is the datum for C3-1, which exhibits essentially no change in $^{87}\text{Sr}/^{86}\text{Sr}$ over 4.56 AE (AE = 10^9 yr).

provided that the Sr was equilibrated with 'bulk meteorite' Sr at that time (Fig. 1). If the CI carbonates and sulphates formed during a period of aqueous activity on the parent body, they would meet this criterion. Indeed, the distribution of Ca in the Orgueil meteorite gives a clue to the behaviour of Sr. Kerridge⁸ has shown that the phyllosilicates which make up 60–65% of the meteorite contain about an order of magnitude less Ca than the bulk meteorite. This suggests that most of the Ca—and by implication Sr—is concentrated in Ca-rich carbonates and sulphates.

Carbonates in the CI meteorites span a large compositional range, and sulphates occur primarily as either gypsum or hydrated magnesium sulphate. Sulphates are present as vein minerals clearly deposited along fractures, while the textural relationship between carbonates and the bulk of the meteorite is much less clear^{4,6}. Richardson⁶, based on a petrographic study of three CI chondrites, claimed that at least three generations of mineralization can be recognized, during which first carbonates, then Ca-sulphates and finally Mg-sulphates were deposited in veins. In the present meteorite, however, only the sulphates exist as true vein minerals; carbonates occur as single crystals or sometimes as aggregates which Richardson⁶ interpreted as residual vein fragments. The most abundant carbonates are dolomite and breunnerite, the latter an Fe-Mg-carbonate. Both of these frequently contain considerable Mn⁹. Pure calcium carbonate occurs only rarely.

Sr isotopic data for carbonate and sulphate-rich samples hand-picked from Orgueil are given in Table 1. Because of the expected low abundances of the elements of interest, and their susceptibility to leaching and contamination, sample processing was kept to a minimum. Bulk meteorite samples were disaggregated ultrasonically in double-distilled acetone or methanol. Light-coloured grains $\geq 20 \mu\text{m}$ were handpicked and grouped according to appearance. Energy-dispersive X-ray analysis using the scanning electron microscope was used to confirm the identity of phases, but because of the small sample amounts, only spot checks could be made and none of the separates is considered to be pure.

Three separate bulk meteorite samples were obtained from P. Pellas (Muséum National d'Histoire Naturelle, Paris) and

Table 1 Sr isotopic data for Orgueil carbonates and sulphates

Sample	$^{87}\text{Sr}/^{86}\text{Sr}$	Sr (p.p.m.)	$^{87}\text{Rb}/^{86}\text{Sr}$
ORG-1			
C1-1 (dolomite)	0.701007 ± 38	70	0.013
C1-2 (mixed carbonate)	0.70200 ± 10	68	0.004
ORG-2			
C2-1 (mixed)	0.70202 ± 28	17	0.44
S2-1 (Ca-sulphate)	0.76785 ± 7	87	0.023
ORG-3			
C3-1 (mixed)	0.69882 ± 20	17.1	*
C3-2 (breunnerite + matrix)	0.70522 ± 20	1.06	0.084
Moore Co. plagioclase†	0.69901		
Bulk Orgueil‡	0.75286	7.85	0.805

Sr isotopic data fractionation corrected to $^{86}\text{Sr}/^{88}\text{Sr} = 0.1194$ and relative to $^{87}\text{Sr}/^{86}\text{Sr} = 0.71025$ for NBS 987 Sr.

* Rb amount indistinguishable from blank (≤ 7.4 pg).

† Initial ratio based on measured value for plagioclase, $^{87}\text{Rb}/^{86}\text{Sr}$ from ref. 15, and 4.56 AE.

‡ After data in ref. 10. The Sr isotopic ratio was calculated using data from Orgueil samples 1 and 3 (ref. 10), our Moore Co. plagioclase initial ratio, and 4.56 AE.

processed as described above. The heterogeneity of the CI meteorites is emphasized by the fact that the populations of light-coloured phases in the three samples varied considerably in mineralogical makeup. In ORG-1, most of the mineral picked as carbonate was dolomite. ORG-2 and ORG-3 both contained considerably more breunnerite than dolomite, and ORG-2 contained a relatively large amount of Ca-sulphate. Each of the mineral separates for which data are reported in Table 1, except for sulphate sample S2-1, was rinsed in double-distilled water in an ultrasonic bath before dissolution; all dissolution was accomplished in 2M HCl and in all cases a small amount of undissolved residue remained. Here we discuss key points of the isotopic data and related sample properties. The details of individual samples will be described more fully elsewhere.

The data in Table 1 show a considerable range in $^{87}\text{Sr}/^{86}\text{Sr}$ for the carbonates, from 0.6988 to 0.7052. The relatively large errors result from small sample sizes, all ≤ 1 mg, and therefore extremely small amounts of Sr, ranging from only 32 ng down to 77 pg. The highest measured value is for Ca-sulphate S2-1, with $^{87}\text{Sr}/^{86}\text{Sr}$ greater than reported values for the bulk meteorite¹⁰ (Table 1). However, the Rb/Sr ratio does not correlate with the Sr isotopic ratios. The lowest measured $^{87}\text{Sr}/^{86}\text{Sr}$ is for sample C3-1, overlapping within its relatively large error limits our internal initial ratio for basaltic achondrites, determined from measurements of Moore Co. plagioclase (Table 1) and an assumed age of 4.56 AE (AE = 10^9 yr). Sample C3-1 was a small mixed breunnerite + dolomite sample. After dissolution, a small amount of low-Mg breunnerite and a few small enstatite grains remained. The bulk of the dissolved material is assumed to have been dolomite + high-Mg breunnerite. Thus these phases must have been deposited very early in the history of Orgueil, perhaps essentially contemporaneously with parent body formation. The measured isotopic ratio is an upper limit because it is possible that a small amount of high $^{87}\text{Sr}/^{86}\text{Sr}$, non-carbonate Sr may have been included; nevertheless it provides an upper limit for the initial CI meteorite Sr isotopic ratio. Rb in C3-1 was indistinguishable from blank levels (≤ 7.4 pg). An important task will be to determine the Sr isotopic ratio more precisely, as other possible indicators of the initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio for carbonaceous chondrites come from refractory inclusions in the Allende CV3 meteorite^{11,12}, and may be representative of neither the bulk Allende meteorite nor carbonaceous chondrites in general. Taking inter-laboratory bias into account, the C3-1 value is consistent with the lowest values reported for Allende^{11,12}.

As mentioned above, other carbonate separates exhibit considerable variability in $^{87}\text{Sr}/^{86}\text{Sr}$, and the values of this ratio do not correlate with $^{87}\text{Rb}/^{86}\text{Sr}$. We do not consider model ages

Table 2 Comparison of water leach and sample data for ORG-3 separates

Sample	$^{87}\text{Sr}/^{86}\text{Sr}$	Sr (pg)	$^{87}\text{Rb}/^{86}\text{Sr}$
C3-1	0.69882 ± 20	647	*
C3-1 leach	0.85337 ± 37	680	0.24
C3-2	0.70522 ± 20	77	0.084
C3-2 leach	0.80592 ± 80	175	1.1

* Rb amount indistinguishable from blank (≤ 7.4 pg).

(see Fig. 1), referred to the bulk meteorite evolution, very meaningful: they range from ~ 4.5 AE to 9 AE using the values from Table 1. We ascribe this to the mobility of Rb in the meteorite, and the consequent difficulty of making accurate measurements of indigenous Rb in the separates, and also to the problem of 'contamination' of the carbonate separates with non-carbonate (bulk meteorite?) Sr. Even the best characterized of our separates, C3-2, which consisted of two large breunnerite crystals, contained small amounts of what appeared to be matrix material injected along cleavage planes. The isotopic data suggest that some of the less well characterized separates were more pure. It is also possible that some of the observed differences are due to real variability in the initial ratios of the carbonates, which could be caused by local (cm-scale) alteration and carbonate deposition and/or variability in the time scale of deposition. Nevertheless, the timing of deposition would have to be very close to the time of parent body formation. If no non-carbonate Rb or Sr were present in the separates for which data are given in Table 1, they must have formed within ~ 200 Myr of parent body formation. Even if all Rb is considered to be extraneous to the carbonates, the time scale is still relatively short—ranging from 540 Myr for sample C3-2, which in fact does contain a matrix contaminant, to 170 Myr for C1-1 and zero for C3-1.

A further important result of these initial data concerns samples C3-1 and C3-2. For both of these, Rb and Sr were measured in the 5-min ultrasonic bathwater rinse. In each case the weight loss for this leach treatment was less than several hundredths of a milligram, that is, less than a few per cent of the total sample. However, the amounts of Sr in the leach solution were about the same (C3-1) and greater than (C3-2) those in the samples (Table 2). In addition, the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio in the leach solutions was very high, considerably greater than that in the bulk meteorite. As in the case of the Ca-sulphate sample, S2-1, the accompanying Rb is insufficient by a large amount to produce this ratio over the lifetime of the meteorite. Thus, there is a mobile, highly radiogenic Sr component in Orgueil not accompanied by its parent Rb. Because of its radiogenic nature, this component must be 'young'. Lacking any knowledge of the Rb-Sr history of the component, it is impossible to quantify this statement, except to say that for Rb/Sr anywhere close to the bulk meteorite, the high $^{87}\text{Sr}/^{86}\text{Sr}$ implies essentially present-day derivation. Times up to a few tens of millions of years would be permitted. The qualitative similarity of the leach and Ca-sulphate data suggests that Sr in the Ca-sulphate may have been derived recently from such a source. These results also emphasize the problem of determining the true initial ratio of the carbonate phases in the presence of such high $^{87}\text{Sr}/^{86}\text{Sr}$ components.

The data for sulphate sample S2-1 are intriguing because they suggest that the calcium sulphate was formed very recently. The timing is uncertain but would include the terrestrial age of Orgueil and the presumed breakup time of the parent body, estimated to be ~ 10 Myr from the cosmic ray exposure age^{13,14}. Most previous workers have suggested, implicitly or explicitly, that the vein-filling sulphates and the carbonates were formed at approximately the same time, and certainly were not separated by billions of years^{4,6}. Sulphate sample S2-1 was not picked directly from veins so that conceivably it is not composed of vein material; however, the platy or sheet-like habit of most of the sulphate fragments implies rather strongly that they were

from fracture linings. Recent dissolution of an early sulphate generation and redeposition incorporating radiogenic Sr could also explain the data. If such an event occurred, it obviously did not greatly affect the carbonates.

These initial experiments show that the Rb-Sr system in Orgueil is complex, but that nevertheless Sr isotopes in the carbonates indicate that they were formed very early, possibly coincident with parent body formation. The lowest measured $^{87}\text{Sr}/^{86}\text{Sr}$ value among our carbonate separates is consistent with published data for the initial Sr isotopic ratio in refractory inclusions from the carbonaceous chondrite Allende^{11,12}. In contrast, the Sr isotopes in a single Ca-sulphate separate suggest that this vein material formed very recently. Further experiments, now underway, will be required to unravel the complicated series of events recorded in the Sr isotopic compositions of the secondary phases of this meteorite.

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Meteoritic $^{138}\text{Ce}/^{142}\text{Ce}$ ratio and its evolution

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The nuclide ^{138}La decays to ^{138}Ce and ^{138}Ba by β^- and electron capture, respectively, with a total half life of about 1×10^5 Myr. Rankama¹ suggested the possibility of age determination using this radioactive decay system. Recently, Tanaka and Masuda² succeeded in age determination by using the ^{138}La – ^{138}Ce geochronometer for a gabbro from the Bushveld complex, South Africa. This La–Ce isotopic system, coupled with the Sm–Nd system, provides a new isotope tracer^{2,3} for the geo- and cosmochemical samples, especially for samples having a Ce anomaly in the rare-earth element patterns. Meteoritic primordial composition of cerium isotopes can serve as a basis for La–Ce systematics. Furthermore, Ce isotopic compositions for meteorites will provide information concerning half-life data of the ^{138}La decay system, which remains uncertain. Here, cerium isotope ratios were determined for Juvinas, Pasamonte and ALH-78132 eucrites and the Jilin (H4) chondrite. Neodymium isotope ratios and abundances of lanthanum, cerium, neodymium and samarium were also determined for these meteorites.

The experimental procedures used are almost identical to those of Tanaka and Masuda². About 0.7 to 3 g of each sample was decomposed. Rare-earth elements (REE) were separated

Table 1 Meteoritic $^{138}\text{Ce}/^{142}\text{Ce}$ and $^{143}\text{Nd}/^{144}\text{Nd}$ data

	$^{138}\text{La}/^{142}\text{Ce}^*$	$^{138}\text{Ce}/^{142}\text{Ce}^\dagger$	$^{147}\text{Sm}/^{144}\text{Nd}^*$	$^{143}\text{Nd}/^{144}\text{Nd}^\dagger$	Primordial $^{138}\text{Ce}/^{142}\text{Ce}^\ddagger$
Jilin(1)	0.003046	0.0228538 $\pm$ 58	0.1921	0.512587 $\pm$ 66	0.0228174 $\pm$ 59
Jilin(2)	0.003128	0.0228521 $\pm$ 114	0.1915	0.512547 $\pm$ 42	0.0228147 $\pm$ 115
Pasamonte	0.003082	0.0228518 $\pm$ 26	—	—	0.0228149 $\pm$ 27
Juvinas	0.003006	0.0228533 $\pm$ 26	—	—	0.0228174 $\pm$ 27
ALH-78132, 71	0.002341	0.0228489 $\pm$ 22	0.1958	0.512543 $\pm$ 24	—
ALH-78132, 78	0.003639	0.0228547 $\pm$ 118	0.1876	0.512440 $\pm$ 30	—
ALH-78132, 79	0.002297	0.0228525 $\pm$ 62	0.1971	0.512587 $\pm$ 32	—

* Uncertainties are less than 0.3%.

† Errors are 2σ mean and correspond to the last significant figures. The $^{138}\text{Ce}/^{142}\text{Ce}$ value for Johnson Matthey reagent, JMC 304, was 0.0228559 ± 0.0000011 (2σ) and the $^{143}\text{Nd}/^{144}\text{Nd}$ value for La Jolla Nd isotope standard was 0.51183 ± 0.00003 (2σ). The average value ($\pm$ s.d.) of the meteoritic $^{138}\text{Ce}/^{142}\text{Ce}$ ratio was calculated by the following equation: $\bar{x} \pm \sigma = [\sum(x_i/\sigma_i^2)/\sum(1/\sigma_i^2)] \pm \sqrt{[\sum(x_i - \bar{x})^2/\sigma_i^2]/(n-1)\sum(1/\sigma_i^2)}$.

‡ The values are estimated for 4.56×10^3 Myr ago, by assuming $\lambda_\beta^{138}\text{La} = 2.58 \times 10^{-12} \text{ yr}^{-1}$ and $\lambda_{\text{EC}}^{138}\text{La} = 4.59 \times 10^{-12} \text{ yr}^{-1}$.

from the major elements by an AG50W-X8 resin column in HCl media, while Ce and Nd were separated using an AG50W-X8 resin column with α -hydroxy-isobutyric acid. Ce and Nd isotopic compositions were measured on a semi-automatic Micromass 54R and abundances of La, Ce, Nd and Sm were determined on a JEOL JMS-05RB mass spectrometer. Cerium isotopes (about 5 μg Ce loaded) were measured as CeO^+ using Re triple filaments and the observed $^{138}\text{Ce}/^{142}\text{Ce}$ were normalized to $^{136}\text{Ce}/^{142}\text{Ce} = 0.01720$. The neodymium ion was measured as NdO^+ using a Re single filament and the measured isotope ratios were normalized against $^{146}\text{Nd}/^{144}\text{Nd} = 0.7219$.

Table 1 shows the results of Ce and Nd isotopic ratios as well as $^{138}\text{La}/^{142}\text{Ce}$ and $^{147}\text{Sm}/^{144}\text{Nd}$ ratios. The $^{138}\text{Ce}/^{142}\text{Ce}$ ratios for Juvinas and Pasamonte eucrites and the Jilin chondrite are between 0.0228518 and 0.0228538. An average value of the present $^{138}\text{Ce}/^{142}\text{Ce}$ ratios for these meteorites is 0.0228527 ± 0.0000009 (2σ). Furthermore, the average value of the $^{138}\text{La}/^{142}\text{Ce}$ ratios for these meteorites is 0.00306 ± 0.00005 (2σ). This value corresponds to 0.378 of the La/Ce abundance ratio. Here, we evaluate the primordial $^{138}\text{Ce}/^{142}\text{Ce}$ ratio for these three meteorites by assuming $T = 4.56 \times 10^3$ Myr, $\lambda_\beta^{138}\text{La} = 2.58 \times 10^{-12} \text{ yr}^{-1}$ and $\lambda_{\text{EC}}^{138}\text{La} = 4.59 \times 10^{-12} \text{ yr}^{-1}$ (ref. 2), and by using the present observed values for the La/Ce abundance ratios. The calculated primordial values of $^{138}\text{Ce}/^{142}\text{Ce}$ are 0.0228174 and 0.0228147 for the Jilin chondrite, 0.0228149 for the Pasamonte eucrite and 0.0228174 for the Juvinas eucrite. The average value of these four calculated ratios for primordial $^{138}\text{Ce}/^{142}\text{Ce}$ is 0.0228162 ± 0.0000014 (2σ). Tanaka and Masuda² estimated primordial $^{138}\text{Ce}/^{142}\text{Ce}$ to be 0.022816. An error in the calculation of primordial $^{138}\text{Ce}/^{142}\text{Ce}$ ratio for each meteorite in Table 1 involves the errors in the isotope and concentration determination. In this calculation, the error in the La/Ce abundance ratio is estimated to be 0.3%. In the calculation, a variation of 0.3% in the $^{138}\text{La}/^{142}\text{Ce}$ value yields only a minor change of 0.0000001 in the primordial $^{138}\text{Ce}/^{142}\text{Ce}$ value. The primordial value (0.0228162) obtained from the Ce isotope ratios of meteorites will be of great significance as a reference for Ce isotope systematics.

The Antarctic eucrites display a heterogeneous distribution of Ce anomaly⁴. Samples ALH-78132,71 and ALH-78132,79 are from the outermost part of the ALH-78132 Antarctic eucrite and they show a positive Ce anomaly. ALH-78132,78 is a chip from the inner part of the same eucrite (several centimetres inside the ALH-78132,79 sample) and this ALH-78132,78 sample possesses a negative Ce anomaly. The observed $^{138}\text{Ce}/^{142}\text{Ce}$ ratios are 0.0228489 ± 0.0000022 (2σ) for ALH-78132,71, 0.0228525 ± 0.0000062 for ALH-78132,79 and 0.0228547 ± 0.0000118 for ALH-78132,78. Based on the primordial value for $^{138}\text{Ce}/^{142}\text{Ce}$, and assuming that the present observed values for the La/Ce abundance ratio are valid for 4.56×10^3 Myr, except for the change due to radioactive decay, one is led to the result that the present $^{138}\text{Ce}/^{142}\text{Ce}$ ratios should be 0.0228442 ± 0.0000015 for ALH-78132,71, $0.0228437 \pm$

0.0000015 for ALH-78132,79 and 0.0228597 ± 0.0000015 for ALH-78132,78. Comparing the observed $^{138}\text{Ce}/^{142}\text{Ce}$ ratios of ALH-78132 with the corresponding values of other meteorites and calculated $^{138}\text{Ce}/^{142}\text{Ce}$ ratios for ALH-78132, we conclude that the positive Ce anomaly observed in ALH-78132,71 and ALH-78132,79 was not produced 4.56×10^3 Myr ago. This conclusion suggests strongly that the positive Ce anomaly in question was produced by terrestrial weathering. The observed $^{138}\text{Ce}/^{142}\text{Ce}$ ratio of ALH-78132,78 is somewhat lower than the calculated ratio, but the precision in isotope ratio determination for this sample was relatively poor. Therefore, more precise determination of Ce isotope abundance will be needed before we know whether the negative Ce anomaly for ALH-78132,78 was produced by terrestrial weathering.

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Carbon isotope composition of low molecular weight hydrocarbons and monocarboxylic acids from Murchison meteorite

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The origin of the organic matter in carbonaceous meteorites remains controversial despite extensive study^{1–4} over the past 20 yr. Motivated by the expectation that the patterns of isotopic variation with molecular structure among the organic compounds would contain important clues to their origin, we have measured the carbon isotopic compositions for individual hydrocarbons and monocarboxylic acids from Murchison meteorite, a C2 carbonaceous chondrite which fell in Australia in 1969. With few exceptions, notably benzene, the volatile products are substantially isotopically heavier than their terrestrial counterparts, signifying their extraterrestrial origin. For both classes of compounds, the ratio of ^{13}C to ^{12}C decreases with increasing carbon number in a roughly parallel manner,

and each carboxylic acid exhibits a higher isotopic ratio than the hydrocarbon containing the same number of carbon atoms. These trends are consistent with the kinetically controlled synthesis of higher homologues from lower ones^{5,6}. The results suggest the possibility that the production mechanisms for hydrocarbons and carboxylic acids may be similar; they also impose constraints on the identity of the reactant species.

Identifications and abundances of individual hydrocarbons^{7,8}, monocarboxylic acids^{9,10}, and amino acids^{11–20} have been reported, but these data have not identified clearly either the source regions or the production mechanisms for these compounds. Large differences in carbon isotopic composition between carbonate and acid insoluble organic matter have been interpreted as evidence for a Fischer–Tropsch synthesis for all the carbonaceous matter from CO, H₂ and NH₃ in the solar nebula^{21,22}. However, recent measurements of the nitrogen^{23–25}, carbon^{3,24–26} and hydrogen^{24–27} isotopic compositions of the organic matter question rather than support this simple interpretation. In a summary of the only previous attempt to measure the isotopic composition of individual compounds, a range of values was reported collectively for an amino acid fraction²⁶.

In a closed, evacuated 700-ml Pyrex apparatus, three interior pieces (23.5 g) of Murchison meteorite were disaggregated by 40 freeze–thaw cycles in water (50 ml) assisted by sonication. After a 20-ml aliquot of the head space gas was removed for analysis of the predominant constituent, carbon dioxide, (Table 1), the remaining head space gases were cryogenically transferred, along with several millilitres of water, to a KOH trap cooled with liquid nitrogen, while the freeze–thaw apparatus was maintained at 40 °C by a water bath. Normally, methane and carbon monoxide are non-condensable at liquid nitrogen temperature, but they become condensable at that temperature in the presence of water, probably by clathrate formation⁸. The gases in the KOH trap were released by warming and introduced into a gas chromatograph–combustion (GCC) system^{28,29}, which separated and combusted each component to carbon dioxide for isotopic analysis. The ¹³C abundances of hydrocarbon standards processed through the isolation–combustion procedure differed by no more than 0–0.2% from those of unprocessed standards. Ethane was the worst case in that it was 0.2% enriched in ¹³C relative to the unprocessed standard. The recoveries of standards were 94–100% with butene having the poorest at 94%. Apparently, little, if any, sample loss or isotopic fractionation occurred during the overall analytical procedure. Abundances and carbon isotope values for the individual hydrocarbons thus obtained from Murchison meteorite are listed in Table 1.

For the analyses of the carboxylic acids, the solution in the freeze–thaw apparatus was separated from the meteorite solid, neutralized with KOH, and dried by rotary evaporation. The resulting residue was redissolved in water, acidified with H₃PO₄ and distilled. The volatile carboxylic acids in the distillate were separated by ion suppression chromatography and combusted to CO₂ in the GCC system. The isotopic compositions of two known carboxylic acids, acetic and propionic acid, were in good agreement (<0.7%) before and after processing through the analytical procedure. The abundances and isotopic compositions of the water soluble carboxylic acids in Murchison meteorite are given in Table 1.

The abundances of carboxylic acids and saturated hydrocarbons decrease with increasing carbon number, and acids are more abundant than hydrocarbons of the same carbon number by factors of 10 (C₃) to 100 (C₂). The carbon in the acids is systematically heavier isotopically than that in the hydrocarbons. Generally, for both classes of compounds, increasing carbon number is also associated with decreasing ¹³C-abundance, and branched isomers are isotopically heavier than their normal (straight-chained) counterparts. The isotopic compositions of the saturated members of each class are plotted against carbon number in Fig. 1, with CO₂ taken as the C₁-acid counterpart to CH₄ and assuming –7.9% (Table 1) to be representative of pentane. Except for the deviation by butane, two roughly parallel curves result for the normal isomers.

Table 1 Abundance and isotopic composition of hydrocarbons* and monocarboxylic acids† in Murchison meteorite

Compound	Abundance (nmol g ⁻¹)	δ ¹³ C _{PDB}
Carbon dioxide	2.4 × 10 ³	+29.1 ± 0.2
Carbon monoxide	2.1	–32 ± 2
Methane	8.9	+9.2 ± 1.0
Ethane	8.5	+3.7 ± 0.1
Propane	8.9	+1.2 ± 0.1
Isobutane	4.3	+4.4 ± 0.1
Butane	5.5	+2.4 ± 0.1
Pentane	†	†
Ethene	1.3	–0.1 ± 0.4
Butenes	†	†
Benzene	28	–28.7 ± 0.2
Acetic acid	1.70 × 10 ³	+22.7 ± 0.2
Propionic acid	6.2 × 10 ²	+17.4 ± 0.2
Isobutyric acid	2.5 × 10 ²	+16.9 ± 0.2
Butyric acid	1.8 × 10 ²	+11.0 ± 0.3
Isovaleric acid	1.0 × 10 ²	+8.0 ± 0.2
Valeric acid	47	+4.5 ± 0.2

* The gas mixture was transferred to a KOH trap at liquid nitrogen temperature. To separate these volatile gases for GCC analysis, the gases from the KOH trap were passed through a trap at dry ice–acetone temperature (water removal) on the way to the GCC sample loop (liquid nitrogen temperature), where the condensable gases collected and the noncondensable gases (mostly methane and carbon monoxide) flowed through to a collection trap with the aid of a Toepler pump. Except for CH₄ and CO, separation of the hydrocarbons on the GCC system was achieved on a Porasil B column. Chromatographic conditions: thermal conductivity detector, stainless steel column (2.5 m × 0.3 cm internal diameter) packed with Porasil B 100/150 mesh. Initial oven temperature, 28 °C, initial time 16 min; temperature programmed at 6 °C min⁻¹ from 28 to 200 °C; flow rate 15 ml min⁻¹. Methane and carbon monoxide were separated on a stainless steel chromatographic column (1 m × 0.3 cm internal diameter) packed with molecular sieve 5A, 100/120 mesh, column temperature, 60 °C, flow rate 15 ml min⁻¹.

† The aqueous acid extract was brought to pH 10 with 2.7 M NaOH and dried by rotary evaporation. The sodium salts were dissolved in 5 ml H₂O, acidified to pH 2 with H₃PO₄ and distilled cryogenically. The distillate was brought to pH 10 and dried at 100 °C. The resulting residue was taken up in 200 µl of 1 M H₂SO₄ and chromatographed on a Hewlett–Packard 1084B liquid chromatograph, equipped with an Alltech RP-8 column (25 cm × 4.6 mm). A gradient of 100% 0.01 M H₂SO₄ going to 85% 0.01 M H₂SO₄ and 15% MeOH was used to separate the acids. The butyric and valeric acids (*n*- and *iso*-) were collected as two groups. The collected fractions were neutralized with NaOH and dried at 100 °C. Each sample was dissolved in 100 µl of H₂O and 50 µl of H₃PO₄ and cryogenically distilled for GCC. The acids are then separated and combusted to CO₂. Gas chromatographic conditions: stainless steel column (2 m × 0.3 cm internal diameter) packed with 10% Alltech AT 1000 on Gas Chrom A 60–80 mesh, oven temperature 140 °C, flow rate 15 ml min⁻¹.

‡ Analysed together, 1.2 × 10⁻² µmol g⁻¹, –7.9 ± 0.05%.

Although these data alone do not give clear indications of the origin(s) of the hydrocarbons and acids, some general conclusions about the required extraterrestrial processes are warranted. Both classes of compounds are products of kinetically controlled processes. That the abundances of the individual hydrocarbons in the suite are approximately of the same magnitude contradicts relative abundances expected of a thermodynamically controlled process. Calculations carried out for sets of temperature, pressure, and H to C to O elemental ratios that approximate possible solar nebular and planetary conditions³⁰ yielded relative abundances for methane, ethane, and benzene that vary typically by factors exceeding 10⁵. The observed variations shown in Table 1 are even smaller than those reported by Des Marais *et al.*³¹ for a similar suite of hydrocarbons in natural gas samples whose kinetically controlled relative abundances differed typically by <10³. A similar conclusion regarding the role of thermodynamic equilibrium is applicable to the carboxylic acid abundances^{30,32}.

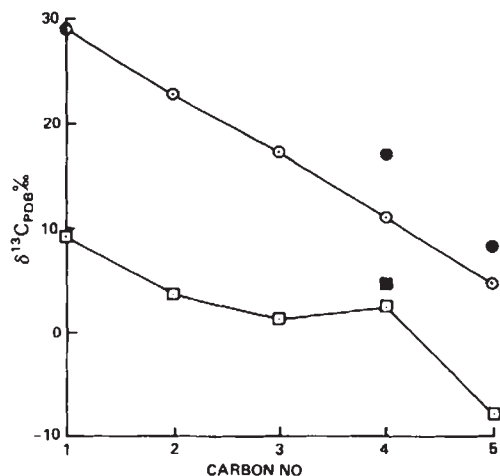


Fig. 1 Plots of $\delta^{13}\text{C}_{\text{PDB}}$ values of individual saturated hydrocarbons (□) and carboxylic acids (○) against carbon number. For example, 1 denotes methane or CO_2 (○), 2 ethane or acetic acid and so forth. Values for branched isomers are indicated by filled symbols.

The variation of isotopic composition with carbon number is consistent with formation of higher molecular weight species from lower homologues. Chang *et al.*⁶ and Des Marais *et al.*⁵ demonstrated that the ratio of ^{13}C to ^{12}C in hydrocarbons synthesized from methane are lower than that of the reactant, decreasing monotonically with increasing carbon number. This pattern follows from the fact that in kinetically controlled synthesis a reactant containing ^{12}C typically reacts more rapidly than its counterpart containing ^{13}C . The trend towards isotopically lighter carbon with increasing carbon number is apparent for both classes of compounds in the data of Table 1. The roughly parallel isotopic variation between both classes implies that some aspects of their histories are similar. The isotopic data also indicate that the carbon monoxide and acid insoluble carbon (both isotopically light, $< -10\%$) are unlikely to have supplied the source carbon for most, if not all, the compounds analysed. No processes have been reported that yield organic compounds in which the carbon is isotopically heavier than that of the starting material. Multiple production mechanisms are not excluded by the data, however. Work in progress is aimed at elucidating the possible connection between CO_2 and the hydrocarbons in the synthesis of the carboxylic acids by determining the intramolecular isotopic composition of the volatile acids.

In principle these compounds in carbonaceous meteorites may have been synthesized in interstellar clouds, the pre-solar nebula or parent bodies (or some combination)^{3,4,23-27,32}. This uncertainty in origin may be diminished as more systematic study is directed at determining the variations of C, N, H-isotopic composition with chemical structure in the organic constituents of meteorites, on the one hand, and in the organic compounds produced by model processes, on the other. When the patterns of isotopic variation with chemical structure in the natural and synthetic products are compared, similarities may come to light that are diagnostic of common production mechanisms and are therefore attributable to specific source regions.

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Stable isotope compositions of gases and vegetation near naturally burning coal

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Our measurements of stable isotope compositions of CO_2 issuing from vents produced by naturally burning coal indicate that the coal is oxidized through a kinetic process in which ground-water is the oxidizing agent. The CO_2 produced by the oxidation of the coal is extremely depleted in ^{13}C relative to normal atmospheric CO_2 . The change in the $\delta^{13}\text{C}$ value of atmospheric CO_2 near the vents resulting from the burning coal was not recorded in tree rings from red cedars, but the $\delta^{13}\text{C}$ values of some C_3 and C_4 type plants collected from within the area were greatly affected. Our results indicate that the $\delta^{13}\text{C}$ values of some species of plants may be sensitive indicators of changes in the carbon isotopic composition of atmospheric CO_2 .

Although the photosynthetic pathway of a plant appears to be the primary factor that determines its carbon isotope ratio^{1,2}, the range in the carbon isotopic composition of plants having the same pathway must be due to other factors. Mazany *et al.*³ suggested that variations in the $^{13}\text{C}/^{12}\text{C}$ ratios of tree rings from the same tree were due to past changes in climate, and that the two species of trees they studied must have had different responses to these climatic changes. Sternberg and DeNiro⁴ measured a range of nearly 5% in the $^{13}\text{C}/^{12}\text{C}$ ratios of cellulose of C_3 plants collected from the same area and they ascribed this range to differences among the biological factors of the plants. Inasmuch as the $^{13}\text{C}/^{12}\text{C}$ ratios measured by Sternberg and DeNiro⁴ were different for the cellulose from plants collected from the same area and having the same photosynthetic pathway and physiognomy, the biological factors must be as species-dependent as the climatic factors.

To understand better the response of different species of plants to changes in the $^{13}\text{C}/^{12}\text{C}$ ratio of atmospheric CO_2 due to the burning of fossil fuels, we have measured the stable isotopic composition of herbaceous plants and trees surrounding vents produced by naturally burning coal as well as of the gases emanating from these vents. Carbon isotope ratios of plants surrounding burning coal vents should be sensitive indicators of

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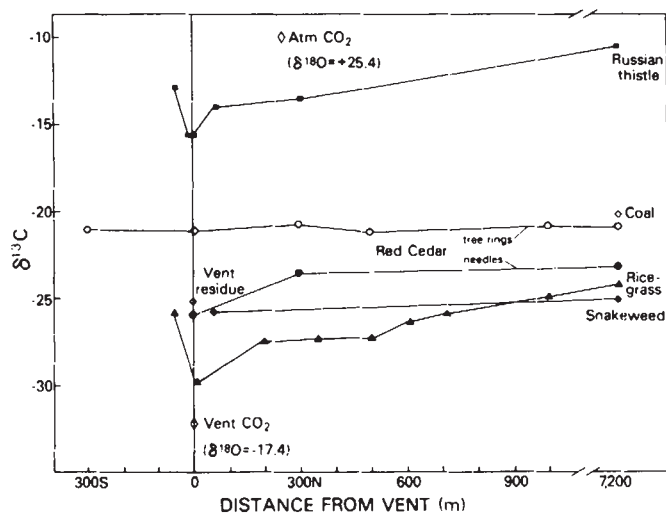


Fig. 1 Variations in $\delta^{13}\text{C}$ values of various materials as a function of their proximity to the vent. CO_2 issuing from the vent has a $\delta^{13}\text{C}$ value of -32.0 and a $\delta^{18}\text{O}$ value of -17.4 , whereas atmospheric CO_2 300 m north of the vent has a $\delta^{13}\text{C}$ value of -10.3 and a $\delta^{18}\text{O}$ value of $+25.4$. Residue collected inside the vent has a $\delta^{13}\text{C}$ value of -25.0 , and coal samples collected away from the vent area at two separate sites have identical $\delta^{13}\text{C}$ values of -21.0 . Wood from tree rings of red cedar exhibits no isotopic effects from burning coal contamination, whereas needles of a red cedar on the vent trend do show some contamination. Roots from Russian thistle (a C_4 plant), stems from ricegrass, and roots from snakeweed (both C_3 plants) exhibit decreasing contamination in their $\delta^{13}\text{C}$ values as a function of their distance north of vents (prevailing wind is from the south). The plants collected south of the vent exhibit very little contamination from the vent gas in their $\delta^{13}\text{C}$ values.

Table 1 Stable isotope compositions of materials and gases associated with naturally burning coal

Sample	Location	$\delta^{13}\text{C}$	$\delta^{18}\text{O}$
Coal	Bed 1*	-21.0	
Coal	Bed 2*	-21.0	
CO_2	Large vent	-32.5	-17.4
CO_2	Atmosphere 300 m north of vents	-10.3	+25.4
Carbonaceous material	Wall of large vent	-25.1	

* Coal beds 1 and 2 are two separate veins collected south of the vent trend from an area where there was no evidence of burning.

the response of different species to local effects of fossil fuel burning because the CO_2 produced from the combustion of coal should be extremely depleted in ^{13}C relative to normal atmospheric CO_2 . In addition, stable isotope ratios of the gases emanating from the vents may provide information on the mechanisms by which coal burns naturally.

Carbon isotope ratios of organic material were determined using carbon dioxide gas that was produced by the complete combustion of the organic material at 800–1,000 °C in purified oxygen at 1 atmosphere. The carbon dioxide was purified and then analysed in a 6''–60'' sector isotope ratio mass spectrometer. Before combustion, the wood samples were chopped in a Wiley mill, then extracted with benzene and ethanol in a Soxhlet extractor to remove soluble components. The resulting sample was composed primarily of cellulose and lignin. Differences between the $^{13}\text{C}/^{12}\text{C}$ ratios of wood treated in this manner and untreated wood were small ($<0.2\%$). The wood samples were collected from tree rings representing the past 20 yr of growth. The height of the trees was 1–2 m. Plant samples were dried and combusted directly, without any chemical treatment. The herbaceous plants were all ~ 0.5 –1 m in height. Atmospheric and vent gases were collected in evacuated stainless steel cylinders and CO_2 was separated and purified by differential freezing

Table 2 $\delta^{13}\text{C}$ values of trees and herbaceous plants

Tree	Distance and direction from main vent	$\delta^{13}\text{C}$
Red cedar (<i>Juniperus scopulorum</i>)	300 m S	-21.0(TR)
	10 m N	-21.3(TR) -25.9(N)
	(on east part of vent trend)	
	300 m N	-21.3(TR) -23.6(N)
	500 m N	-21.4(TR)
Herb	1,000 m N	-20.8(TR)
	7,200 m NW (control)	-21.0(TR) -23.2(N)
Russian thistle (<i>Salsola kali</i> L.)	1 m S	-15.5(R) -16.8(L)
	50 m SW	-12.4(R)
	2 m N	-15.6(R) -17.4(L)
	50 m N	-13.5(R) -14.1(L)
	300 m N	-13.1(R)
Ricegrass (<i>Oryzopsis webberi</i>)	7,200 m NW (control)	-11.0(R) -12.4(L)
	2 m S	-25.8(ST) -24.9(L)
		-29.6(R)
	2 m W	-29.7(ST)
	100 m W	-27.6(ST)
	2 m E	-30.4(ST)
	100 m NE	-26.1(ST)
	1 m N	-29.9(ST) -30.5(R)
	200 m N	-27.5(ST)
	325 m N	-27.5(ST) -25.8(L)
Snakeweed (<i>Gutierrezia sarothrae</i>)		-28.4(R)
	500 m N	-27.3(ST)
	600 m N	-26.4(ST)
	700 m N	-26.1(ST)
	1,000 m N	-25.2(ST)
	7,200 m NW (control)	-24.8(ST) -24.7(L)
		-24.7(R)
	50 m N	-25.7(R) -24.7(L)
	50 m NW (control)	-25.2(R) -24.2(L)

TR, Tree rings; N, needles; R, roots; L, leaf; ST, stem.

techniques. The results are reported in units of ‰ relative to SMOW standard for oxygen or relative to PDB standard for carbon and are accurate to $\pm 0.2\%$ based on replicate analysis. The $\delta^{13}\text{C}$ value of NBS-21 in this laboratory is -28.1 relative to PDB.

The burning coal beds are part of the Straight Cliffs Formation located at 37°17'30" N, 111°22' W in the Escalante Quadrangle near the Utah–Arizona border. This formation consists of interbedded, Upper Cretaceous marine and nonmarine sandstones, mudstones, shales and coal⁵. Areas where coal is burning or has burned are easily recognized because the sandstones, which are usually yellow, are turned red by the heat and gases from the burning coal. The collection site is located on the southern edge of the arid Kaiparowits Plateau in the Smoky Mountains. A 200-m long, east–west-trending series of small vents each ~ 4 cm in diameter, breach the red sandstone surface of this area. Two large vents (1–2 m in diameter) on the western edge of this trend are the most active and, therefore, served as the focus of the study. The local topography was such that the vents were ~ 2 m higher in elevation than the surrounding area. Approximately 10 l min^{-1} of steam and carbonaceous gases issue from each of the large vents. The gas collected from the vents consists of $\sim 70\%$ air, 7% steam, 20% CO_2 , 2% CH_4 and 1% saturated hydrocarbons. These gases are blown northwards by prevailing winds.

Coal from different beds in this area have $\delta^{13}\text{C}$ values near -21.0 (Table 1, Fig. 1). Carbonaceous material which precipitated on the walls of the vents has a lighter value of -25.1 and CO_2 from the vent is even lighter at -32.5 (Table 1, Fig. 1). Modern atmospheric CO_2 normally has $\delta^{13}\text{C}$ values between -7.5 and 8.0 (ref. 6 and I. Friedman and J.D.G., in preparation), so that the CO_2 issuing from the vent is not atmospheric but is derived from the oxidation of the coal. Furthermore, equilibrium fractionation factors indicate that ^{13}C is always concentrated in

Table 3 Calculated carbon isotope fractionations between certain plant species and atmospheric CO₂

Common name	Species	Organ	$\Delta^{13}\text{C}$ plant-CO ₂
Ricegrass	<i>Oryzopsis webberi</i>	Stem	-17
Russian thistle	<i>Salsola kali</i>	Root	-3

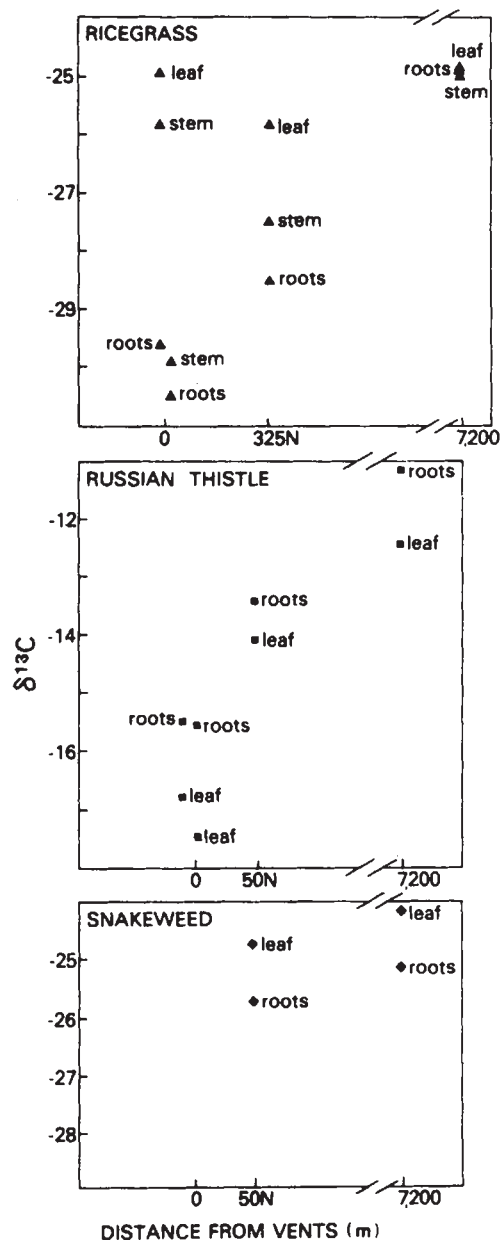


Fig. 2 $\delta^{13}\text{C}$ values of various parts of plants as a function of their proximity to the vents. Snakeweed and ricegrass, both C₃ plants, have more negative $\delta^{13}\text{C}$ values in their roots than in their leaves or stems, whereas Russian thistle, a C₄ plant, shows the inverse trend. Although the difference between the $\delta^{13}\text{C}$ values of roots and leaves from Russian thistle is constant, those plants growing near the vents have $\delta^{13}\text{C}$ values which indicate that they have incorporated vent CO₂ during photosynthesis. Ricegrass exhibits large variations among the $\delta^{13}\text{C}$ values of various organs of plants collected in areas where there is contamination from vent CO₂, and very little variation in organs from plants collected from the control area. The roots of ricegrass collected south of the vent (prevailing wind is from the south) exhibit contamination by vent CO₂, whereas the leaf and stem do not.

CO₂ relative to a reduced-carbon phase even at high temperatures⁷ so that the CO₂ from the vent is not in isotopic equilibrium with either the carbon in the coal or the carbonaceous material from the walls of the vent. Instead, the isotope data indicate that the coal burns by a kinetic process wherein ¹²C in the coal is preferentially oxidized relative to ¹³C.

The oxygen isotopic composition of the vent CO₂ is -17.4 (Table 1, Fig. 1), a value only moderately more ¹⁸O-depleted than the isotopic composition of -15 for groundwater that would be expected from this area⁸. Atmospheric O₂ has a $\delta^{18}\text{O}$ value of +23.5 (ref. 9). The extreme ¹⁸O depletion of the vent CO₂ relative to that expected from the kinetic oxidation of the coal by atmospheric O₂ leads us to conclude that the source of most of the oxygen in the naturally burning coal must come from a reservoir having a low $\delta^{18}\text{O}$ value such as that of the groundwater.

Red cedars (*Juniperus scopulorum* Sarg.) were collected with the anticipation that the ¹³C-depleted CO₂ issuing from vents would produce wood having low $\sigma^{13}\text{C}$ values. The $\delta^{13}\text{C}$ values of wood from trees 5 and 10 m north of the vents were identical to the values of control trees >7.2 km away (Table 2, Fig. 1). Evidence that the red cedars were exposed to the vent CO₂ is observed in the $\delta^{13}\text{C}$ values of some of the needles of a cedar located 10 m north of the main vent (Fig. 1). These were depleted in $\delta^{13}\text{C}$ by 2‰ relative to needles from trees further from the vents, suggesting that they may be more sensitive indicators of local isotopic anomalies than are the tree rings. The absence of low $\delta^{13}\text{C}$ values of the tree rings from samples collected near the vents suggests that the fractionation of carbon isotopes between red cedars and atmospheric CO₂ is not always constant.

Unlike the red cedars, the isotopic compositions of certain herbaceous plants were affected by the CO₂ issuing from the burning coal vents (Table 2, Fig. 1). The effect was observed in the carbon isotopic compositions of plants using each of the two common pathways of photosynthesis and in plants with different physiognomies. For example, the $\delta^{13}\text{C}$ values of samples of Russian thistle, a herbaceous annual C₃ plant, and ricegrass, a C₄ plant, are both strongly dependent on their position relative to the vents. Samples of snakeweed, a woody perennial C₃ plant, have $\delta^{13}\text{C}$ values which are less dependent on their position relative to the vents.

Although different organs of each plant have different carbon isotopic compositions because of variations in their chemical components¹⁰, the ¹³C/¹²C ratio of the vent CO₂ is reflected in the $\delta^{13}\text{C}$ values of all the organs in plants collected near the vents (Fig. 2). Differences among the $\delta^{13}\text{C}$ values of the organs of snakeweed and Russian thistle are constant although organs of these plants closest to the vents are depleted in ¹³C relative to corresponding organs of the control plant. However, the difference between the roots of snakeweed collected 50 m north of the vents is depleted in ¹³C by only 0.5‰ relative to the roots of the control plant whereas the roots of Russian thistle 50 m north of the vents is depleted by 2.5‰ relative to the control plant. Differences among the $\delta^{13}\text{C}$ values of various organs of ricegrass are not constant: the roots and stems have more variable $\delta^{13}\text{C}$ values than do the leaves.

Russian thistle and ricegrass appear to be the species most sensitive to changes in the isotopic composition of local atmospheric CO₂. With a $\delta^{13}\text{C}$ value of atmospheric CO₂ of -8.0 surrounding the control plants, the fractionation factor between the roots of Russian thistle (C₃ plant) and CO₂ is -3.0 whereas the fractionation factor between the stems of ricegrass (C₄ plant) and CO₂ is -16.8. Atmospheric CO₂ collected 300 m north of the vents above the zone that may have been affected by CO₂ from the local flora and fauna has a $\delta^{13}\text{C}$ value of -10.3 (Table 1, Fig. 1). The fractionation factor between roots of Russian thistle collected 300 m north of the vents and the local atmospheric CO₂ having a $\delta^{13}\text{C}$ of -10.3 is -2.8 whereas the corresponding factor between the stems of the ricegrass 325 m north of the vent and this CO₂ is -17.2. Although the fractionation factors for these two species differ greatly, the fractionation

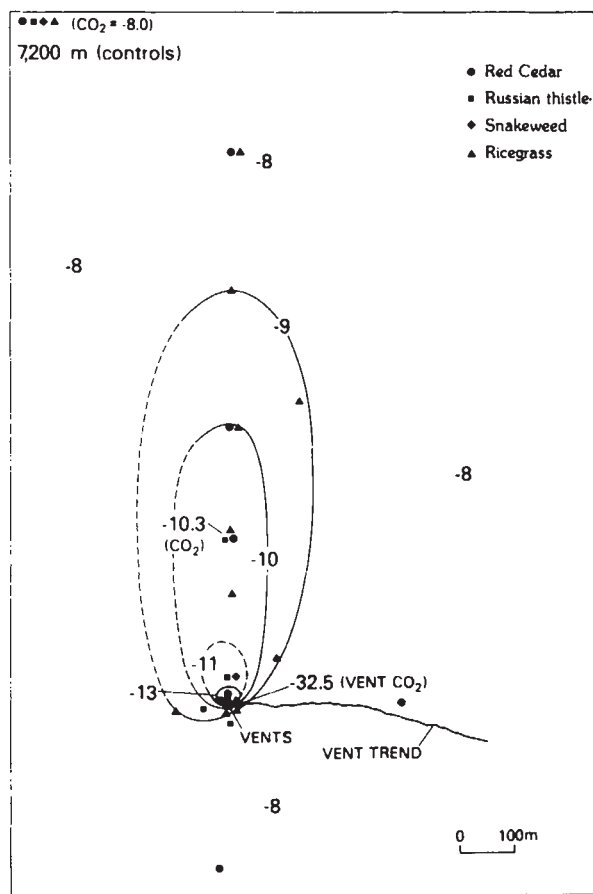


Fig. 3 Estimated $\delta^{13}\text{C}$ contours of local atmospheric CO_2 in the area surrounding the vents. From a $\delta^{13}\text{C}$ value of -8.0 for CO_2 in equilibrium with the roots of Russian thistle and stems of ricegrass from the control area and a value of -10.3 for atmospheric CO_2 in equilibrium with these plants collected 300 m north of the vents, the fractionation of $\delta^{13}\text{C}$ between the organs of these plants and atmospheric CO_2 was calculated (Table 3). By applying these calculated isotope fractionations to the $\delta^{13}\text{C}$ values measured in plants collected from the area surrounding the vents, contours can be drawn showing average $\delta^{13}\text{C}$ values of atmospheric CO_2 . The $\delta^{13}\text{C}$ values of -32.5 for vent CO_2 and -10.3 for atmospheric CO_2 collected 300 m north of the vents are also shown.

factors between the control plants and local CO_2 are similar to those of plants of the same species under the influence of ^{13}C -depleted CO_2 from the vents. This suggests that the $\delta^{13}\text{C}$ values of Russian thistle and ricegrass reflect the isotopic composition of the local atmospheric CO_2 .

Because the $\delta^{13}\text{C}$ values of tissues of Russian thistle and ricegrass depend on the $\delta^{13}\text{C}$ value of atmospheric CO_2 surrounding the plants during photosynthesis, it should conversely be true that the $\delta^{13}\text{C}$ value of atmospheric CO_2 can be determined from the $\delta^{13}\text{C}$ value of the plant. By using the fractionation factors listed in Table 3 and the $\delta^{13}\text{C}$ values of the plants from the area around the vents, $\delta^{13}\text{C}$ contours of atmospheric CO_2 surrounding the vents have been estimated (Fig. 3). Contamination effects of the vent CO_2 on the isotopic composition of the plants are detected up to a distance of 1 km to the north.

The burning coal area located in the Escalante Quadrangle near the Utah-Arizona border is a natural laboratory for studying the effects of the burning of fossil fuels on the atmosphere and on the biosphere. Our results suggest that: (1) the source of most of the oxygen in gases from naturally burning coal may be from a low $\delta^{18}\text{O}$ reservoir such as groundwater; (2) wood from tree rings does not necessarily record small variations in the $\delta^{13}\text{C}$ values of atmospheric CO_2 ; and (3) some C_4 and C_3 plants do record small variations in the value of atmospheric CO_2 so that by analysing the $\delta^{13}\text{C}$ value of selected organs of

some carefully chosen plants, the extent of any contamination to normal atmospheric CO_2 can be estimated.

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Persistence of organic phosphates in buried soils

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Studies of the occurrence of phosphate in soil on archaeological sites have sometimes measured total phosphate¹, but more frequently the inorganic or 'available' phosphate^{2,3}—that extractable by brief treatment with acid or by inorganic solutions in the cold. The nature of the organic phosphate component does not seem to have been examined. Here we present an analysis of total phosphate and inorganic phosphate in 50 samples of soil taken from various levels of a site with a continuous settlement record from 1,600 to 6,800 yr BP. We could detect no mineralization of organic phosphate, at least over the period 1,600–5,200 yr BP. But in the upper soils, corresponding to the interval 1600–2800 BP, there was much more organic phosphate than in the lower samples, the transition being quite sharp. We tentatively conclude that the lower soils belonged to an island site relatively sparsely inhabited and often covered with natural vegetation. The upper soils corresponded to a more dense settlement in which organic phosphate, possibly phytic acid, was deposited on the transient surface and was not incorporated into the soil phosphate cycle.

Three groups of workers have compared the proportions of organic and inorganic phosphate in soil buried beneath an ancient structure: a Roman villa in England⁴ (~1,800 yr BP), a megalithic tomb in Ireland⁵, (~4,000 yr BP), and a Kurgan burial mound in Moldavia⁶ (~4,500 yr BP). In each instance the findings were essentially similar. When the phosphate content of soil layers just below the present-day soil surface were compared with equivalent layers below the buried structure (a layer 25–50 cm below the surface was used for comparison in order to eliminate any phosphate that might have been added by human activity such as a burial), it was found that the total phosphate content of the two soil layers was not markedly different, but the organic phosphate content had markedly decreased. In the present-day soil samples the organic phosphate was about 30–40% of the total, a value which is characteristic of many soils⁷, while in the buried soil the organic phosphate fraction was only 10–15%. As far as can be determined from these three observations, the extent to which organic phosphate had been lost does not seem to depend on the length of time that the soil had been buried. Possibly partial hydrolysis took place quite quickly⁸, but the occlusion was in each case a single event, and it is not possible to say anything about the kinetics.

The collection of a large number of soil samples from a 2 × 2 m sondage excavated in a Yugoslav tell site has enabled phosphate analyses to be made on soils which have been buried for various times, thus making it possible in principle to plot the progress of mineralization against time. The site, tell Gomolava on the Sava River about 80 km west of Belgrade, lies in a district of

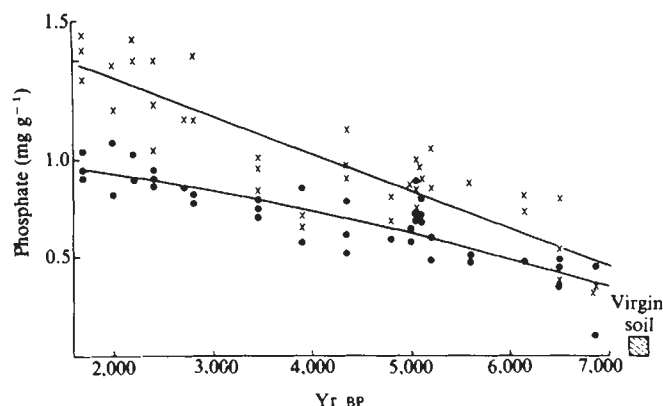


Fig. 1 Total phosphate (× and upper line) and inorganic phosphate (● and lower line) in soil samples from tell Gomolava, Yugoslavia. The lines show the best fit to the points predicted by an optimization routine. The basis of the model used for fitting is described in the text.

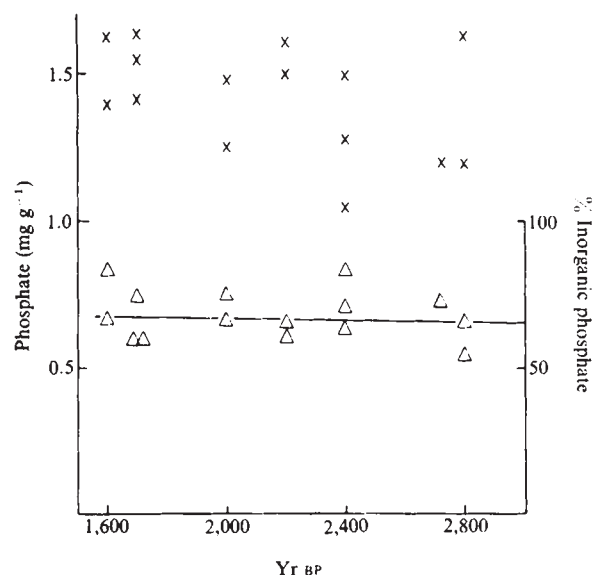


Fig. 2 Total phosphate (×) and inorganic phosphate as per cent of the total (Δ) over the period 1,600–2,800 yr BP at tell Gomolava. The line is the regression of the percentage points with time. It is not significantly different from zero, and the intercept lies at 66% (= 34% organic phosphate).

calcareous loess (pH 8–8.5), so that it is possible completely to exclude leaching. Unlike the three sites mentioned above, the total phosphate content of the buried virgin soil is very low, about 0.15 mg g^{-1} , so that almost all the phosphate in the archaeological samples must have come directly or indirectly from human activity. This has introduced complications in the interpretation of the results, but there are several points of interest that do not appear in a straight comparison of natural soils. The sondage was taken down about 6 m to virgin soil; the layer immediately above this is estimated to have been deposited in the early Vinča-Pločnik period (about 6,850 yr BP). The tell was occupied more or less continuously until sub-Roman times, so that the samples span a range of rather more than 5,000 calendar years (1,600–6,850 yr BP). The dating of the layers, by radiocarbon dating and by traditional archaeological methods (for example, by ceramic typology and by coin dating in the La Tène and Roman eras) is discussed in detail elsewhere⁸. It can be regarded as fairly reliable.

The analytical methods have been described earlier¹. Total phosphate was measured by a nitrate incineration procedure, and inorganic phosphate by a 15-min extraction with 2 M HCl at 100 °C. About 130 soil samples were assayed for total phosphate. Approximately 50, selected to cover all the 19 archaeological strata, each about 30 cm deep, were chosen at random within the replicate samples available for each layer and assayed for inorganic phosphate. Organic phosphate was taken as the difference between the two measurements on a single soil sample.

The overall results for total and inorganic phosphate, for the soil samples that were subjected to both analyses, are shown in Fig. 1. Because the occupation density was markedly greater in Iron Age and Roman times than in the Neolithic, the total phosphate content of the excavated soil decreases with age, although even in the earliest Vinča-Pločnik layers it is clearly greater than that of the buried virgin soil, as indicated by the block in the lower right-hand corner of Fig. 1. (No measurements for present-day 'virgin' soil are available because the entire surrounding district is now heavily cultivated.) This variation makes it difficult to interpret changes in the relative inorganic phosphate content of soil samples of different ages. A simple mathematical model, with a straight line fitted to the total phosphate points, and assuming that the fraction of organic phosphate would be the same no matter when the soil was deposited, but that the organic fraction would be mineralized at a rate proportional to its concentration in the soil, produced the lower curve in Fig. 1. This fits the data reasonably well, but it gives a half-life for the organic phosphate of ~7,000 yr, with a decay constant that cannot be statistically distinguished from zero. This implies an infinite life for the organic phosphate fraction. Sections of the complete data were therefore examined in more detail (Figs 2, 3).

Figure 2 shows the results for the period 1,600–2,800 yr BP, during which time the total phosphate content of the samples was reasonably constant, averaging $1.3 \text{ mg P per g dry soil}$. It can be seen that over this 1,200-yr period there is no evidence for mineralization. The inorganic phosphate content if anything decreased slightly with increasing age of sample. The same picture emerges from Fig. 3, which covers the period from 3,450 to 5,200 yr BP. (Allocation of the data to Figs 2 and 3 was dictated primarily by the lower total phosphate content of the samples shown in Fig. 3, averaging 0.9 mg P per g , but it corresponds also to a change in the nature of the settlements on the site, and of the palaeobotanical evidence, which is discussed in detail below.)

Consideration of Figs 2 and 3 suggests that over a total period of almost 4,000 yr, there is no evidence for conversion of the remanent organic phosphate into inorganic phosphate. It is however remarkable that the average organic phosphate content of the soils dated between 1,600 and 2,800 yr (Fig. 2) is about 34%, while that of the older samples (Fig. 3) is only about 12%. The difference is very consistent, and suggests that the two sets of soil samples had somewhat different histories, almost certainly before they were buried. Possible reasons for the difference are now discussed.

The transition point corresponds to the archaeological transition between Eneolithic and Copper Age cultures on the site (for example Baden and Kostalač) to full Bronze Age cultures (Vučedol, followed later by the Iron Age, Hallstatt and later cultures). It also corresponds almost exactly with a massive increase in the number of cereal seeds (mostly millet, einkorn and barley⁹) obtained from the sondage layers by flotation. It seems likely from the two pieces of evidence that there was a change from a subsistence still partly supplemented by hunting and fishing to one overwhelmingly based on cereal agriculture. A high proportion of the seeds found in the upper layers were charred, and thus brittle, so that one may realistically assume that the deposited soil will, from 3,000 yr BP onwards, contain a greater proportion of abraded grain than in earlier levels, whether the abrasion was caused by treading underfoot or by grinding into flour. The major organic phosphate compounds present in seeds, especially cereal seeds, are *myo*-inositol polyphosphates, principally phytic acid⁷. It is generally agreed that this class of compound is very stable in natural soil; it is thus reasonable to suppose that an important component of the organic phosphate fraction of the soil samples from the period

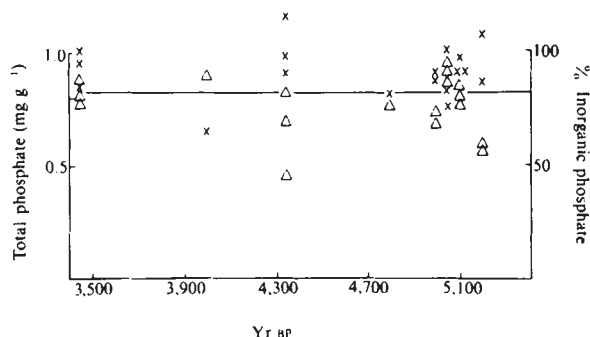


Fig. 3 Total phosphate (x) and inorganic phosphate as per cent of the total (Δ) over the period 3,400–5,600 yr BP at tell Gomolava. The line has the same significance as in Fig. 2, and the intercept lies at 88% (equivalent to 12% organic phosphate).

1,600–2,800 BP at tell Gomolava is comprised of inositol polyphosphates. As Fig. 2 provides no evidence of mineralization, it may be presumed that inositol polyphosphates are indeed completely resistant to biological attack in the conditions in which they were preserved. They would have been buried rather quickly, by collapse of mudbrick walls and the like¹¹, under as much as 30 cm of soil, on which a new phase of the settlement was then established. If confirmation can be obtained by direct analysis phytate would become an important addition to the list of resistant organic compounds whose identification can be of assistance to archaeologists and palaeoecologists.

In discussing the possible reasons for the lower proportion of organic phosphate found in the earlier buried soils, the results shown in Fig. 3, conclusions must be more tentative, because the argument has to be by analogy. There is no way of establishing what was the organic phosphate content of those soils when they were buried. One can only say that it must have been much higher in absolute amount than that of the buried virgin soil, because the organic phosphate content of the former is even now as great as the total phosphate of the latter. If the original organic phosphate of the soils buried earlier than ~3,000 yr BP did not come predominantly from cereal grains, it must nevertheless in some way have been synthesized. Almost certainly much of it was synthesized from the phosphate, largely inorganic, added to the soil by human and animal excretion. Except in the very earliest habitation levels on the site, the total phosphate of the soil was sixfold greater than that of the virgin soil. The soil phosphate cycle is sufficiently well understood¹⁰ for it to be extremely probable that the organic components were synthesized as the result of a symbiosis between plant growth and soil microbial activity. If this reasoning is correct, the original organic phosphate may well have been considerably higher than 12% of the total, with fairly rapid mineralization of much of it. Gardiner and Walsh⁵ concluded that this had been the case, both in the soil that they studied, and in the English buried soil⁴.

This interpretation of events is of interest because it implies that until the middle of the fourth millennium the site was covered, at least in part, by vegetation. This idea is attractive for the following reasons. The density of settlement was much less in Neolithic times than later, and it seems unlikely that the whole tell surface, originally some 2–3 hectares in extent, was continuously occupied. It has been suggested that parts of the tell were in Neolithic times covered by local vegetation for quite long periods⁹. It is even possible that some cereal culture was carried out on the tell itself, as it was originally surrounded by extensive marshes bordering the Sava. The composition of the buried virgin soil, and of the clay exposed on the river bank, suggests that phosphate may have been rate-limiting for growth, so that vegetation would have grown rapidly when the tell surface received deposits of phosphate and also of nitrogen, from the settlers and their domestic animals.

This analysis of the results therefore suggests two phases in the development of the tell. In an earlier phase the tell resembled

an island slightly raised above the surrounding marsh, and patchily settled by people who raised the fertility of the soil but did not prevent it from metabolizing phosphates in a way characteristic of normal soils. This was followed by a later phase in which settlement was much more intense, so that vegetation was necessarily much sparser. In addition, it seems possible that in the later phase, pre-formed organic phosphates (probably phytate) may have been deposited on the transient surface by changes in dietary and agricultural habits, but were not incorporated into the soil structure. This analysis agrees closely with what has already been deduced about the history of the tell from geological and palaeoecological observations^{9,12}. The transition between the occurrence of shade-loving and open-country snails at the end of the Neolithic⁹ is particularly significant in this regard.

It may be felt that this interpretation of the analytical results depends very heavily on inference and analogy. It is, however, susceptible to test in the following way. Although the reasons are not understood, it is known that the inositol in phytic acid is entirely the *myo*-isomer⁷, while that in the inositol polyphosphates extracted from a variety of natural soils contains a mixture of the *myo*-, *chiro*-, *scyllo*- and *neo*-isomers of inositol^{10,13}. Thus extraction of the inositol polyphosphates from earlier and later soil samples from tell Gomolava, followed by identification of the isomers, should make it possible to demonstrate whether the inositol in the later soils came predominantly from cereal grains, while that in the earlier soils has the isomeric composition characteristic of a stable natural soil, even though it is now part of an artificial mound. A programme of research to this end has been initiated in this laboratory.

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Presence of NADPH-cytochrome P450 reductase in central catecholaminergic neurones

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The cytochrome P450-containing mixed function oxidases metabolize a variety of endogenous and exogenous compounds including drugs, carcinogens, fatty acids and steroids¹. Mixed function oxidases have been detected in several tissues², including brain³. The enzyme system consists of a lipid fraction (phosphatidylcholine), cytochrome P450 and NADPH-cytochrome P450 reductase⁴. NADPH-cytochrome P450 reductase has been purified to apparent homogeneity and demonstrated to supply reducing equivalents from NADPH to cytochrome P450 (refs 5–7). Detection of NADPH-cytochrome P450 reductase thus represents an indirect means of demonstrating the presence

of cytochrome P450. Although the role of cytochrome P450 in the central nervous system (CNS) is not known, it may include such different functions as metabolism of xenobiotics⁸, aromatization of androgens to oestrogens⁹ and the formation of catecholestrogens¹⁰. Despite the potentially very important role(s) of cytochrome P450 in brain function, its exact regional distribution remains essentially unknown. Using a specific antibody against rat liver NADPH-cytochrome P450 reductase in combination with immunohistochemical techniques, we have now localized this enzyme to defined catecholamine (CA)-containing structures of the rat and monkey brain.

NADPH-cytochrome P450 reductase purified from rat liver microsomes according to the method of Guengerich and Martin¹¹ with minor modifications¹², was >95% pure as judged by SDS-polyacrylamide gel electrophoresis¹³. Protein concentrations were determined by the method of Lowry *et al.*¹⁴. Antibodies to the enzyme were raised in rabbits and IgG fractions were isolated from the serum by affinity chromatography using a protein A-Sepharose CL-4B column¹⁵, then concentrated to contain 25 mg protein ml⁻¹. IgG was isolated in a similar way from preimmune serum. The IgG fractions were dialysed against phosphate-buffered saline (PBS; 10 mM potassium phosphate buffer pH 7.4, containing 0.15 M NaCl). The antibodies were specific for the antigen as judged by the following criteria¹²: inhibition of NADPH-cytochrome *c* reductase activity of the purified antigen and in rat liver microsomes; Ouchterlony immunodiffusion; and immunoblotting¹⁶ (described in Fig. 3 legend).

For the immunohistochemical studies, 15 rats (Sprague-Dawley, 180 g; Anticimex, Stockholm) of both sexes and two female monkeys (Talapoim, 1 kg) were perfused with 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), their

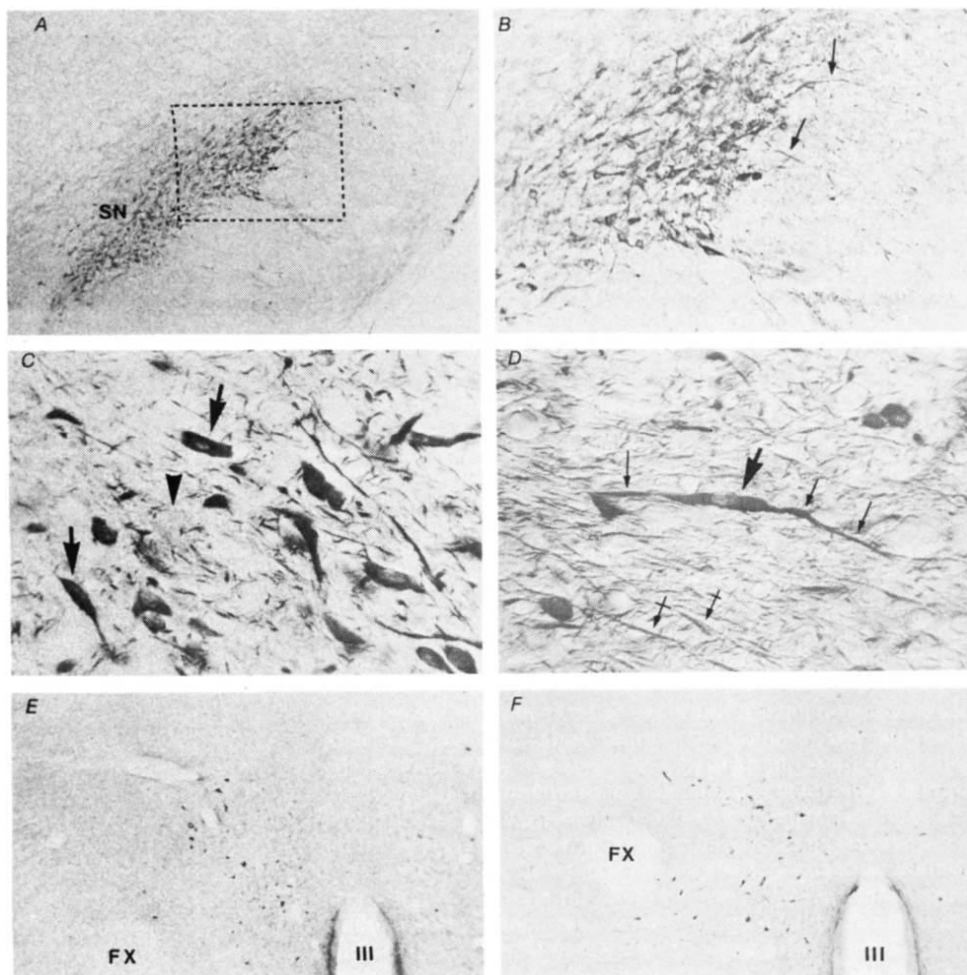
brains removed and cut in a cryostat (at 10 μ m thickness) or on a Vibratome (at 70 μ m). The sections were incubated with anti-NADPH-cytochrome P450 reductase antibody (diluted 1:300 to 1:1,000 in PBS containing 0.3% Triton X-100) for 1–6 days and the antibody–antigen complex visualized using either the immunofluorescence method of Coons¹⁷ or the avidin–biotin complex method of Hsu *et al.*¹⁸. In a separate study of the possible coexistence of NADPH-cytochrome P450 reductase and tyrosine hydroxylase (TH) within the same neurone, sections were incubated with either antibody and reacted with 4-chloronaphthol according to the methods of Nakane¹⁹, Gu *et al.*²⁰ and Tramu *et al.*²¹ (Fig. 2).

Neurons containing a strong NADPH-cytochrome P450 reductase-like immunoreactivity are present within neuronal cell bodies of the following brain areas: the substantia nigra (SN), the ventral tegmental area of Tsai, the rostral part of dorsal raphe, the nucleus locus coeruleus and the ventrolateral medulla region of both rats and monkeys. NADPH-cytochrome P450 reductase-positive neurones are present also in the periventricular nucleus of the hypothalamus, the nucleus arcuatus (including the tanycytes of this nucleus) and the medial aspect of the zona incerta (Fig. 1).

All the brain areas listed above are known to harbour neurones containing the catecholamines dopamine (DA), noradrenaline (NA) or adrenaline (A)²².

As the catecholamines are dependent on TH for their synthesis²³, alternating sections were incubated with a well characterized anti-TH antibody^{24,25}. Analysis of serial sections revealed an overlapping distribution of cell bodies containing TH- and NADPH-cytochrome P450 reductase-like immunoreactivity in the mes- and diencephalon and nerve terminals in the following forebrain regions: the striatum, nucleus accum-

Fig. 1 A–F, Photomicrographs showing neurones in the substantia nigra (A–D) and hypothalamus (E, F) containing strong NADPH-cytochrome P450 reductase-like immunoreactivity. A large number of NADPH-cytochrome P450 reductase-positive cell bodies are present throughout the SN of the rat (A). The photomicrograph in B is an enlarged view of the enclosed area in A; arrows mark dendrites containing immunoreactivity. C and D show NADPH-cytochrome P450 reductase-positive neurones in pars compacta (arrows in C) and reticulata (large arrow in D) of the monkey SN. Small arrows in D indicate dendrites, and crossed arrows indicate dendrites of pars compacta cells that extend into reticulata. The substantia nigra of both rats and monkeys contains numerous cells lacking NADPH-cytochrome P450 reductase-like immunoreactivity (arrowhead in C). Incubations of sections in preimmune IgG or anti-NADPH-cytochrome P450 reductase IgG preabsorbed with the pure antigen (100 μ g per 1,000 μ l diluted antibody at 4 °C for 24 h) resulted in no staining in any of the brain regions examined. E and F show NADPH-cytochrome P450 reductase-positive neurones in the hypothalamus after incubation with the antibody alone (E) or antibody preabsorbed with pure TH (150 μ g per 1,000 μ l diluted antibody at 4 °C for 24 h (F). Note that preabsorption with TH did not block the staining of neurones with NADPH-cytochrome P450 reductase antibody. The peroxidase–anti-peroxidase method was used. Nomarski phase interference contrast for A–D. A, E, F, $\times 56$; B, $\times 230$; C, D, $\times 360$. FX, fornix; III, third ventricle.



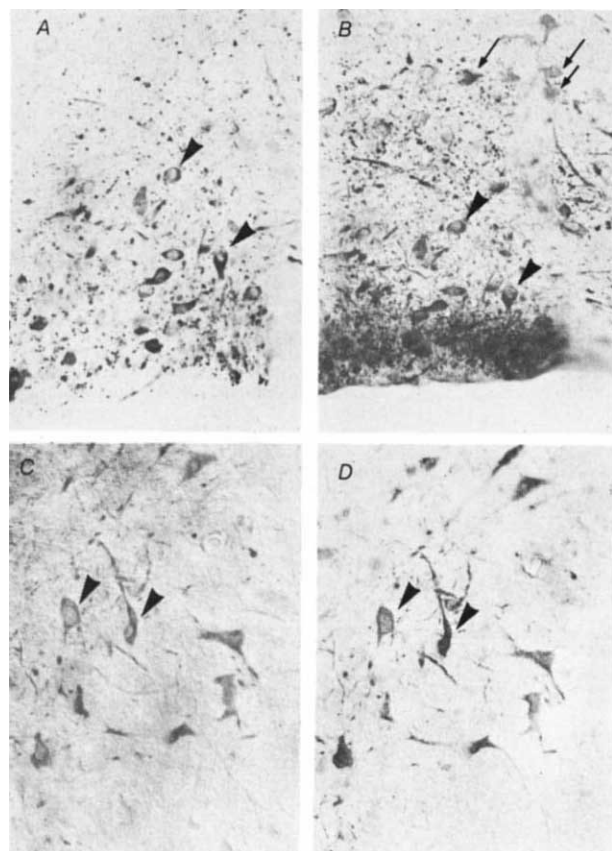


Fig. 2 (A–D), Photomicrographs of neurones in the ventral tegmental area of Tsai (A, B) and the pars reticulata of the substantia nigra (C, D) in rat, demonstrating the simultaneous localization of TH (A, C) and NADPH-cytochrome P450 reductase-like immunoreactivity (B, D) within the same neurones.

Methods: Vibratome sections (70 μ m) were incubated for 7 days in anti-TH antibody (diluted 1:2,000) and the antibody-antigen complex visualized using the avidin-biotin complex method with 4-chloronaphthol as a chromogen. The stained sections were coverslipped in PBS and glycerol (1:3) and the areas containing TH-like immunoreactivity photographed. The sections were subsequently decolorized in alcohol, eluted according to the method of Tramu *et al.*²¹ and re-incubated in NADPH-cytochrome P450 reductase IgG (diluted 1:2000) for 5 days. The sections were reacted with 3', 3'-diaminobenzidine and H_2O_2 to produce a brown reaction product. The stained sections were defatted, coverslipped in Pertex, re-photographed and analysed for the presence of double-labelled cells. Large arrowheads indicate cells containing both antigens. Small arrows in B mark cells containing NADPH-cytochrome P450 reductase but lacking TH-like immunoreactivity. Nomarski optics. $\times 500$.

bens, olfactory tubercle and the septum. Lesions of the DA-containing axons ascending from the SN to the striatum in the lateral hypothalamus by infusions of the catecholamine neurotoxin 6-hydroxydopamine (6-OHDA, 8 μ g per 4 μ l; survival time of 10 days) resulted in a loss of NADPH-cytochrome P450 reductase and TH-like immunoreactivity from the striatum, accompanied by an increased intensity of immunoreactivity in the cell bodies of the SN ipsilateral to the lesion. These experiments suggest a coexistence of NADPH-cytochrome P450 reductase and TH in dopaminergic neurones of the SN. In separate experiments where the anti-NADPH-cytochrome P450 reductase antibody was preabsorbed with pure TH before incubation of the sections, staining of neurones in the SN and hypothalamus occurred to the same extent as after incubations with non-absorbed antiserum. Taken together, these findings show that the anti-NADPH-cytochrome P450 reductase antibody does not cross-react with TH and suggest that NADPH-cytochrome P450 reductase and TH occur in the same population of neurones. This hypothesis was confirmed by direct double



Fig. 3 Autoradiogram of an immunoblot showing the presence of NADPH-cytochrome P450 reductase (Red) in a microsomal fraction isolated from rat brain. Microsomes were isolated from the substantia nigra, ventral tegmental area of Tsai, striatum, nucleus accumbens and olfactory tubercle. Microsomes were prepared according to the procedure described by Holtzman and Desautel³¹. Immunoblotting was performed by the method of Towbin *et al.*¹⁶ with minor modifications¹². SDS-polyacrylamide gel electrophoresis¹³ was performed using 8% acrylamide and 2.7 mm-thick slab gels. Proteins were transferred to nitrocellulose sheets which were then incubated for 2 h with antibody (or preimmune IgG) against rat liver NADPH-cytochrome P450 reductase (IgG fraction). The immunoreactive bands were visualized on the nitrocellulose filters using ¹²⁵I-labelled protein A followed by autoradiography. In the above experiment 0.1 μ g of purified NADPH-cytochrome P450 reductase and 42 μ g of brain microsomal protein (Mic) were run on the gel. The top part of the nitrocellulose filter was incubated with anti-NADPH-cytochrome P450 reductase IgG whereas the lower part was incubated with preimmune IgG. NADPH-cytochrome P450 reductase having a molecular weight similar to purified rat liver NADPH-cytochrome P450 reductase was detected in brain microsomes.

labelling experiments in which the same neurone in the SN (and hypothalamus) was found to contain both NADPH-cytochrome P450 reductase and TH-like immunoreactivity. A closer inspection revealed that while most, if not all, TH-positive cells contained NADPH-cytochrome P450 reductase, many reductase-positive neurones contained no TH immunoreactivity (Fig. 2).

The present study represents the first histochemical demonstration of NADPH-cytochrome P450 reductase in brain. The presence of NADPH-cytochrome P450 reductase-like immunoreactivity in central neurones supports earlier biochemical findings of this enzyme in brain²⁶. Our study further extends the biochemical findings by identifying those neurones in the rat and monkey brain that contain the NADPH-cytochrome P450 reductase and thus possibly also cytochrome P450. It seems unlikely that the presence of NADPH-cytochrome P450 reductase and TH-like immunoreactivity in the same cell is due to cross-reactivity of the antibodies as preabsorption of anti-NADPH-cytochrome P450 reductase IgG with excess pure TH (and, conversely, anti-TH antiserum with excess pure NADPH-cytochrome P450 reductase) failed to block the subsequent immunostaining of central neurones.

Figure 3 shows that a protein having the same apparent molecular weight as the purified rat liver NADPH-cytochrome P450 reductase was found in brain microsomes. The part of the immunoblot which was incubated with preimmune IgG showed no reaction. This experiment supports the immunohistochemical data indicating the presence of NADPH-cytochrome P450 in the above-mentioned areas of the brain.

The presence of NADPH-cytochrome P450 reductase immunoreactivity in TH-positive cells probably indicates the existence of a cytochrome P450 system within these neurones. This is of particular interest as cytochrome P450 has been implicated in the formation of catecholestrogens. The conversion of oestradiol and oestrone to catecholestrogens has been demonstrated in the CNS of rat²⁷ and it is probable that the enzyme responsible for this conversion in the brain is similar to the cytochrome P450 found in peripheral tissue¹⁰. The action of the catecholestrogens in the CNS has been linked to their interaction with biogenic amines as competitive inhibitors of catechol-O-methyltransferase (COMT)²⁸ and as possible inhibitors of TH²⁹ although it could also imply that catecholamines are being synthesized through an alternative pathway involving cytochrome P450.

Although the presence of NADPH-cytochrome P450 reductase in central catecholaminergic neurones strongly indicates

the existence of cytochrome P450 within these neurones, it cannot be excluded that this enzyme may have functions not directly linked to cytochrome P450. This could involve the donation of electrons from NADPH to cytochrome b_5 —another electron transfer component identified in brain microsomes³⁰. It has also been proposed²⁶ that NADPH-cytochrome P450 reductase could be involved in the formation of the catecholamine neurotoxin 6-OHDA. It may be speculated that the presence of large amounts of NADPH-cytochrome P450 reductase in central dopaminergic neurones may be of relevance for the formation of endogenous 6-OHDA in the development of neurodegenerative disorders such as Parkinson's disease.

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Effect of impulse blockage on cytochrome oxidase activity in monkey visual system

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Cytochrome oxidase (cytochrome c oxidase; ferrocytochrome c : oxygen oxidoreductase, EC 1.9.2.1) has been introduced as an oxidative metabolic marker for neurones in the central nervous system¹. Previous studies have shown that mature neurones remained sensitive to altered functional demands^{2–9}, and that both developing and adult neurones responded to sensory deprivation or deafferentation by reducing their cytochrome oxidase (Cyt.Ox.) activity^{1,3,4,9–11}. More recently, we showed that the blockage of retinal impulse transmission with tetrodotoxin led to a reversible reduction in Cyt.Ox. staining of affected lateral geniculate (LGN) and striate neurones in adult cats⁹. The present study sought to extend these findings to adult monkeys, where Cyt.Ox. 'puffs' or 'blobs' are uniquely present in the visual cortex^{10,12–14}. We found that, while the retina remained histologically intact, with only moderate decreases in Cyt.Ox. staining of large ganglion cells and the two plexiform layers, subtle changes occurred in the LGN as early as 1 day post-tetrodotoxin injection, and clear reduction in enzyme levels was evident in both the LGN and the visual cortex by 3 days. Changes became progressively more severe up to 4 weeks post-injection. Within area 17, alternating bands of high and low Cyt.Ox. staining occurred in lamina IV, with alternating rows of dark and lightly reactive puffs superimposed in exact register. Thus, the mature visual neurones in the primate remain extremely sensitive to the cessation of retinal impulse transmission, and plastic metabolic changes occur through several synapses along the sensory pathway.

Seven adult monkeys (*Macaca mulatta*) were used; six were experimental and one, plus a normal *Macaca fascicularis*, served as controls. The experimental monkeys were all anaesthetized with ketamine-HCl (15–30 mg per kg intramuscularly), and each received a unilateral intravitreal injection of tetrodotoxin (TTX, 19 µg in 10 µl of sterile water, a dosage found not to block axoplasmic transport)⁹. The pupillary light reflex was again used as a convenient indicator of the impulse blockage effect of TTX which, in the macaque, lasted for at least 4 days per injection. Injections were repeated twice weekly where applicable, and

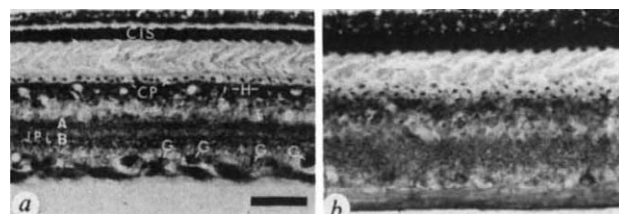


Fig. 1 Transverse sections through the normal (a) and TTX-injected (for 2 weeks) (b) retinae of a macaque. Sections have been reacted for cytochrome oxidase without counterstaining. In the normal retina, note the dense staining of cone inner segments (CIS), cone pedicles (CP), horizontal cells (H) and scattered large ganglion cells (G, arrows). The moderately stained inner plexiform layer (IPL) is subdivided into A and B, with B being slightly more reactive than A and exhibiting two thin bands of reactivity. The TTX-treated retina shows slightly decreased staining of the outer and inner plexiform layers and of the large ganglion cells. Scale bar, 50 µm.

survivals were 1, 3, 6, 14 and 28 days. The animals were then perfused and all eyes and brains were frozen sectioned and processed for Cyt.Ox. histochemistry¹.

The normal retinae of monkeys (as well as those of cats, ferrets and other mammals^{15,16}) exhibited a distinct pattern of Cyt.Ox staining (Fig. 1a). From the photoreceptor layer inward, we found a highly reactive band of cone inner segments, a row of reactive cone pedicles and a row of reactive horizontal cell bodies with some moderately reactive bipolar cells. The inner plexiform layer contained a moderately reactive sublamina A and a slightly more reactive sublamina B, which included two thin reactive bands separated by a relatively clear zone. Finally, the large ganglion cells tended to be the most reactive, and they were scattered amongst slightly less reactive medium and small ganglion cells. The ganglion cell axons were reactive until they formed the optic nerve bundle, at which point they acquired myelin and lost their intense Cyt.Ox. activity.

In the normal macaque LGN, the two magnocellular layers and lamina 6 showed the highest level of Cyt.Ox. activity for both neurones and neuropil, with lamina 5 being moderately reactive and laminae 3 and 4 the least reactive (Fig. 2a) (see also ref. 15). Due to the greater cellular density in lamina 1, it appeared slightly more reactive than lamina 2. The S layer and the interlamellar zones contained relatively non-reactive neuropil and small neurones with occasional moderately reactive medium-sized neurones. In addition, all of the major cellular layers along the medial and lateral boundaries of the LGN, representing more peripheral portions of the visual field¹⁷,

Fig. 2 Transverse sections through the macaque LGN reacted for Cyt.Ox. In the normal LGN (*a*), magnocellular laminae 1 and 2 and parvocellular lamina 6 are the most reactive. Lamina 5 is moderately reactive, while laminae 3 and 4 are the least reactive. After TTX injections, laminae 1, 4 and 6 that are contralateral, and 2, 3 and 5 that are ipsilateral to the injected eye exhibit decreased Cyt.Ox. activity. This pattern, shown here as ipsilateral to the injected eyes, can be seen by 3 days post-injection (*b*), but becomes more severe by week 4 (*c*). Scale bar, 1 mm.

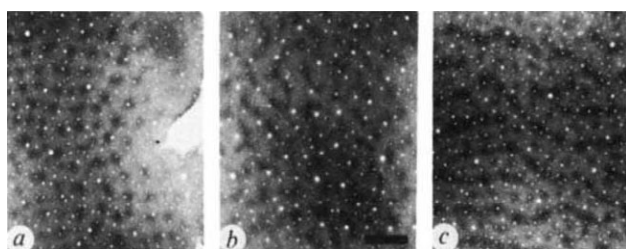
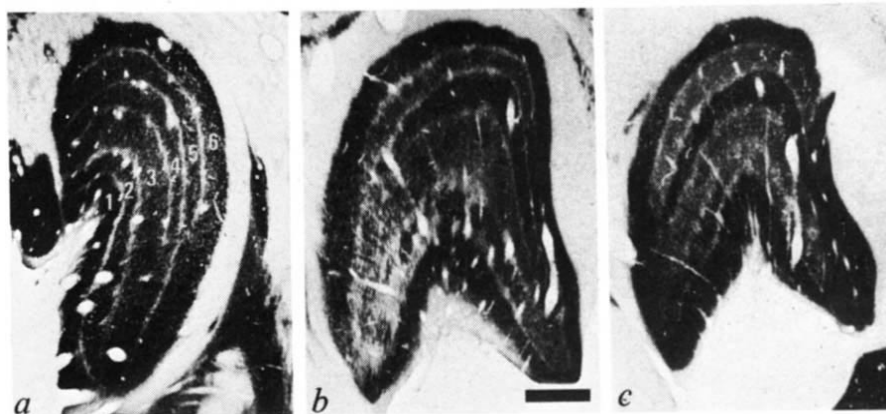


Fig. 3 Tangential sections through Cyt.Ox.-rich puffs in laminae II-III of macaque striate cortex reacted for Cyt.Ox. *a* Shows the normal distribution (see text). After 3 days of TTX treatment (*b*), a slight decrease in enzyme staining can be seen in some of the puffs which have been traced to be in register with lightly reactive bands in lamina IVC. After 4 weeks of TTX treatment (*c*), distinct rows of lightly reactive and shrunken puffs can be seen alternating with rows of darkly reactive puffs. These rows have been traced to line up in exact register with lightly reactive and darkly reactive bands respectively in lamina IVC (see Fig. 5). Scale bar, 1 mm.

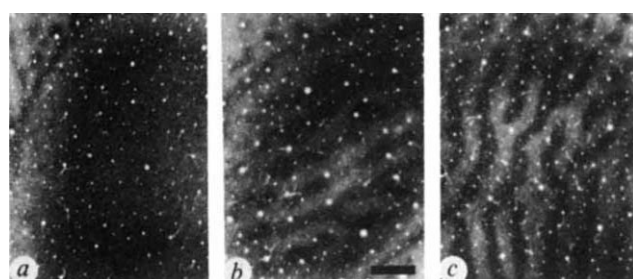


Fig. 4 Tangential sections through lamina IVC of macaque striate cortex reacted for Cyt.Ox. *a* Shows a homogeneous distribution of enzyme activity in the normal monkey. After 3 days of TTX injections (*b*), alternating bands of high and low Cyt.Ox. activity can be seen in IVC. This pattern becomes more distinct after 4 weeks of TTX injections (*c*). Scale bar, 1 mm.

exhibited a higher level of Cyt.Ox. activity than the middle portion of the nucleus.

The pattern of Cyt.Ox. staining in the normal striate cortex of the macaque was similar to that reported previously^{10,18-20}. In the supragranular layers, the Cyt.Ox.-rich zones or puffs^{13,14} were globular in shape, measuring $\sim 262 \mu\text{m} \times 377 \mu\text{m}$ (Fig. 3*a*). The puffs lined up in rows roughly orthogonal to the 17/18 border. The centre-to-centre spacing of puffs within the rows was $450 \mu\text{m}$, while those between rows was about $353 \mu\text{m}$. Lamina IV was also reactive (Fig. 4*a*), with lamina IVA having an intermittent punctate appearance, lamina IVC α being slightly more reactive than lamina IVC β , and the lower edge of lamina IVC β forming a thin band of darker staining. Laminae IVB and V were relatively non-reactive, while lamina VI was again moderately reactive. Reactive neurones, mainly of the stellate type, were seen in all of the cellular laminae. Reactive pyramidal cells were found mainly in laminae II-III, V and upper VI. These findings were comparable with our results in squirrel monkeys^{3,14}.

The effect of TTX on the retina was relatively mild. Even after 4 weeks of injections, the retina appeared remarkably intact histologically, and the pattern of staining remained essentially the same as normal. The major change was a decrease in the level of reactivity of large ganglion cells as well as those of the outer and inner plexiform layers (Fig. 1*b*).

As early as 1 day after TTX treatment, a mild decrease in the level of Cyt.Ox. staining was seen in laminae 2, 3 and 5 of the ipsilateral LGN, while laminae 1, 4 and 6 of the contralateral LGN appeared relatively normal. By 3 days changes were seen in all of the laminae innervated by the TTX-treated eye (Fig. 2*b*) and the severity increased with longer survivals. Because laminae 1 and 6 were normally quite reactive, they were the last ones to exhibit a level of Cyt.Ox. staining that was lower than that of non-TTX-treated laminae. After 4 weeks of TTX

injections, distinct differences were seen between reactive and TTX-affected laminae (Fig. 2*c*). At all survival periods, the ipsilateral laminae suffered a greater reduction of Cyt.Ox. than the contralateral ones.

TTX treatment for 1 day did not reveal significant alterations in enzyme staining or overall dimensions of laminae II-III puffs in the striate cortex. Changes in lamina IV were subtle, with minor fluctuations in staining within lamina IVC. By 3 days, however, alternating bands of high and low Cyt.Ox. activity were clearly visible within lamina IVC (Fig. 4*b*). The puffs exhibited some disarray, with slightly shrunken, paler puffs scattered amidst darkly reactive ones (Fig. 3*b*). Serial tracings of tangential sections (see Fig. 5 legend) confirmed that rows of reactive puffs stayed within the confines of reactive bands in lamina IVC, while rows of light puffs fell within those of light bands in lamina IVC. This pattern of alternating dark and light puffs/bands became more prominent with longer survivals up to 4 weeks (Figs 3*c*, 4*c*, 5). Moreover, light puffs appeared progressively smaller. In register with the pattern in the upper layers, laminae V and especially lamina VI showed alternating bands of dark and light Cyt.Ox. reactivity. At each survival time, the overall dimensions of reactive puffs were comparable with those in control monkeys. However, even at 3 day survival, there was a significant difference ($P < 0.001$) in cross-sectional areas between reactive and non-reactive puffs ($56,372.211 \mu\text{m}^2$ compared with $39,870.51 \mu\text{m}^2$). By 4 weeks, this difference was even more pronounced ($55,504.347 \mu\text{m}^2$ and $17,859.168 \mu\text{m}^2$, $P < 0.001$). On the other hand, the centre-to-centre spacing between reactive puffs of the same row, between non-reactive puffs of the same row, and between puffs of adjacent rows remained essentially the same as the control.

The possibility that these transneuronal and trans-transneuronal enzymatic alterations might be due to retinal damage was deemed unlikely because: (1) All TTX-injected eyes

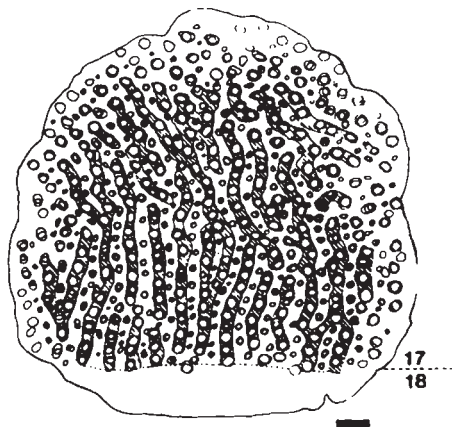


Fig. 5 Composite camera lucida tracings through nine serial tangential sections (spanning a depth of 550 μm) from area 17 of a macaque whose contralateral eye has been injected with TTX for 4 weeks. Note that the large, darkly reactive puffs lie in exact register with darkly reactive bands (hatched) in lamina IVC, while the shrunken, lightly reactive puffs are superimposed over lightly reactive bands in lamina IVC. Scale bar, 1 mm. Tracings such as this were done in the control as well as 11 of the experimental hemispheres. For each hemisphere, the areas of at least 100 puffs overlying the reactive bands, and 100 overlying the non-reactive bands in lamina IVC of each composite tracing were digitized with the aid of a computer. Statistical analyses were done for the mean areas of reactive and non-reactive puffs, and comparisons made between these two groups at different survival periods.

remained histologically intact, similar to the control; (2) the ganglion cells of the injected eyes retained their ability readily to transport tritium-labelled tracers to the LGN (our unpublished observations); (3) the observed response in the striate cortex was rapid (within 3 days and after only a single injection) and widespread rather than localized; and (4) our findings in the cat⁹ and our preliminary results in the monkey indicated that these enzymatic adjustments were reversible. Moreover, histochemical changes appeared to be more dramatic in the brain itself than in the retina (see note added in proof).

The present study thus indicates that the mature primate visual system remains extremely sensitive to the cessation of retinal impulse transmission. Tetrodotoxin is known to cause altered functional properties in the developing kitten visual system^{21,22}, but the adult sensory system was thought to be refractory to the detrimental effect of sensory deprivation. Our present results demonstrate that, not only are changes pronounced at both the geniculate and cortical levels, but the swift onset of transneuronal and trans-transneuronal responses is much more dramatic than our earlier findings in the cat (1 week)⁹, and of reported geniculate cell shrinkage and cortical enzyme changes after a more drastic procedure of enucleation (10 days)^{11,23,24}. The primate visual neurones, then, respond briskly to the blockage of retinal input, even while the system remains structurally intact, and axoplasmic transport is retained. The metabolic changes do not occur uniformly throughout the LGN. Neurones which receive ipsilateral input appear to suffer the consequences slightly earlier and to a greater extent than those receiving contralateral input, as we have observed in the cat⁹. Likewise, neurones representing the central portion of the visual field seem to be more affected than the peripheral ones. Changes in cortical lamina IVC and the puffs indicate that their energy-coupled activities are very much controlled by retinal input. The dark and light bandings in lamina IVC correspond in spacing and dimensions to ocular dominance columns described by Hubel's group²⁵, and are the known sites for geniculocortical terminations²⁶⁻²⁸. The sources of input to the Cyt.Ox. puffs have been postulated to arise from the inter-laminar and S layers of the LGN^{29,30}, although other inputs have not yet been ruled out. The histochemical 'shrinkage' of puffs suggests that, although the entire puff may suffer an overall

lowered level of activity, there may be an additional tendency for the response to progress in a peripheral-to-central direction. In conclusion, we have demonstrated the dramatic and early response of mature primate neurones to the blockage of primary afferent impulses, indicating that the normal functional and enzymatic integrity of cortical neurones are very much governed by activity originating one to several synapses away.

Note added in proof: Our recent findings indicate that 2 weeks of unilateral intravitreal saline injections did not result in any histochemical changes in the visual system of the macaque.

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Community food webs have scale-invariant structure

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We have analysed 62 community food webs drawn from published studies and have found a remarkable regularity in ecosystem structure: in biological communities, the proportions of top, intermediate and basal species are, on average, independent of the total number of species. Hence, there is a direct proportionality between the numbers of prey and predators.

The finding¹ that, in community food webs, the ratio of prey to predators is 3:4 may be challenged on two grounds: first, it is based on a relatively small set of 14 webs, and second it may indicate that taxonomists have exercised greater taxonomic refinement in classifying organisms at higher than at lower trophic levels².

A community food web involves the feeding, that is, trophic, relations among all organisms found in a well-defined habitat by the original investigator. Organisms are separated into 'trophic species', which may be a single biological species, or a size class or stage in the life cycle of a single biological species, or a collection of functionally or taxonomically related biological species, according to the original report. Throughout this paper a 'species' refers to a 'trophic species', not necessarily to a single

biological species. A 'top' species is a predator that has no predator. An 'intermediate' species is a species that is both a predator and a prey. A 'basal' species is a prey that has no prey.

The community food webs analysed include 40 webs assembled and described by Briand³; of these, 13 are corrected and drawn from the 14 originally used by Cohen¹. Details of the food webs not yet presented by Cohen⁴ or Briand³ will be published elsewhere (F.B., in preparation). We find that the number of prey is roughly proportional to the number of predators with a slope less than 1 (Fig. 1a). This is also true for webs from constant and fluctuating environments, although they are quite different in overall structure³. On the far right of Fig. 1a, four outliers emerge from the general relationship: a cluster of three constant food webs (C), all from Fryer's study⁵ of littoral communities of Lake Nyasa, and one fluctuating food web (F) representing a salt meadow from New Zealand⁶.

There can be no direct test of Pimm's conjecture² of why the slope is less than 1, without repeating the original field studies with a uniform attention to taxonomic detail. As an indirect test, we examined the ratio of prey to predators in the 62 food webs, after we had 'lumped' trophically identical species. That is, in each food web, whenever two or more species are preyed on by exactly the same set of predators, and prey upon exactly the same set of prey, we treated them as one. This procedure, which we call 'lumping', removes possible differences in the propensity to split, both among observers and among trophic levels.

Lumping moves the outliers into or much closer to the bulk of the remaining data points, for both fluctuating and constant webs (Fig. 1b). The correlation coefficient between numbers of predators and prey among the 43 fluctuating webs increases from 0.83 before lumping to 0.92 after, and from 0.58 to 0.64 among the 19 constant webs. In other words, eliminating predators or prey that are trophically identical tightens the relation between numbers of predators and prey.

Because individual observers tend to influence prey-predator ratios, we shall deal only with the lumped version of the webs. Because some observers contributed more than one food web to our collection, the assumption of independence that is required to justify attaching probability values to significance tests using the unlumped data is open to challenge. Though the assumption of independence is probably more acceptable with the lumped version of the webs, we shall base our statistical analysis primarily on descriptive statistics. Fortunately the patterns in the data are clear.

If a straight line, either with arbitrary intercept or through the origin, is fitted to a scatter plot of the 62 community food webs, the squared residuals increase with the number of predators. This reveals that the usual least-squares procedure, which assumes the variance of residuals constant regardless of the abscissa, is not appropriate to these data.

If a regression line is fitted through the origin on the assumption that the variance of the residuals is proportional to the number of predators, then the estimator of the slope is simply the ratio of the mean number of prey divided by the mean number of predators. Under this assumption, a straight line through the origin fitted to all 62 food webs has slope 0.8819 or approximately 0.9.

This slope is higher than the slope near 0.75 found by Cohen¹, so there appears to be some merit in Pimm's suggestion² that ecologists have exercised greater taxonomic refinement at high trophic levels than at low. This suggestion, however, is not quantitatively sufficient to account for the excess, that remains after lumping, in the number of predators over the number of prey.

Classical ecological theory views predators as generally limited by resources, and the diversity of predators in particular as being limited by the diversity of prey. From this perspective it would seem more natural to treat the number of prey as an independent variable and the number of predators as a dependent variable. However, when the number of predators is regressed against the number of prey, using a straight line

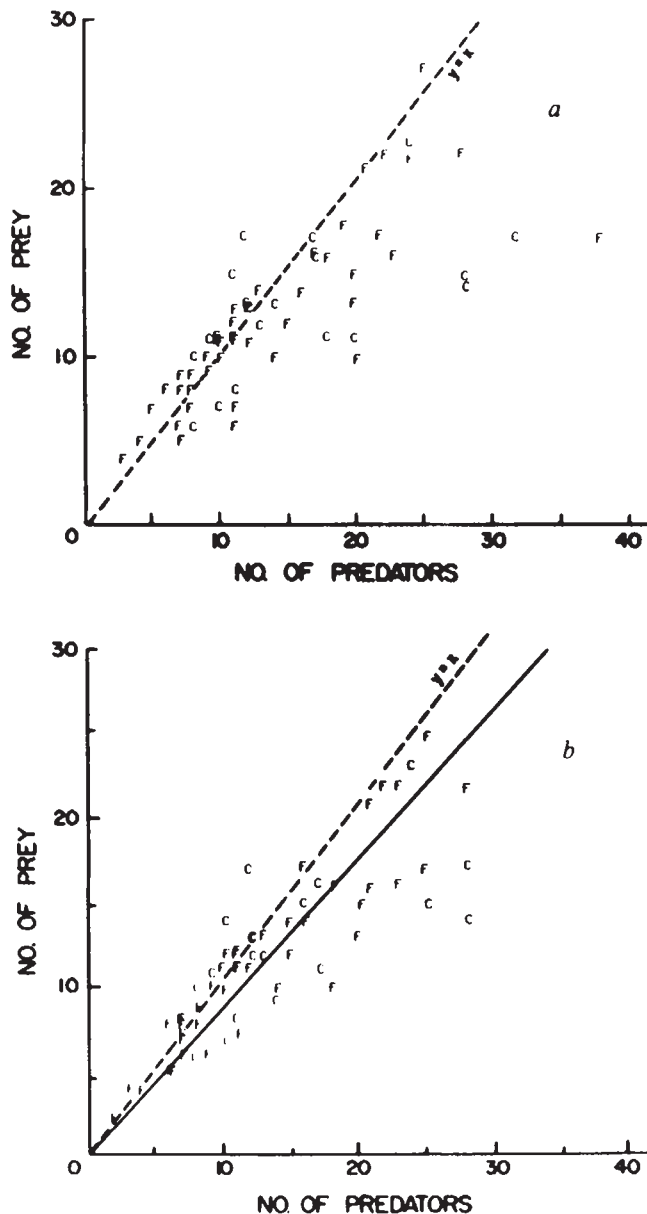


Fig. 1 Number of prey species as a function of number of predator species in 62 community food webs. F, fluctuating environment. C, constant environment. An environment is classified as 'fluctuating' if the original report indicates temporal variations of substantial magnitude in temperature, salinity, water availability, or any other major physical parameter. The magnitude, and not the predictability, of the fluctuations is the criterion of classification. The symbols F and C have been shifted from their exact locations by a small random amount to indicate when several food webs have exactly the same coordinates. a, Original data; b, after lumping. The solid line through the origin is fitted on the assumption that the variance of the residuals is proportional to the number of predators. The slope is 0.8819.

through the origin with variance of residuals proportional to the abscissa, the standard error of estimate (with 61 degrees of freedom) increases from 3.1 to 3.5.

This observation means that the number of predators is a better predictor of the number of prey than the reverse. This raises the intriguing possibility that, in both constant and fluctuating environments, the number of predators is causally more important in controlling the number of prey than vice versa. Evidence and theory in favour of this suggestion have been independently reviewed by Jeffries and Lawton⁷.

That we find a linear relationship between number of prey and number of predators is not too surprising, since the x- and

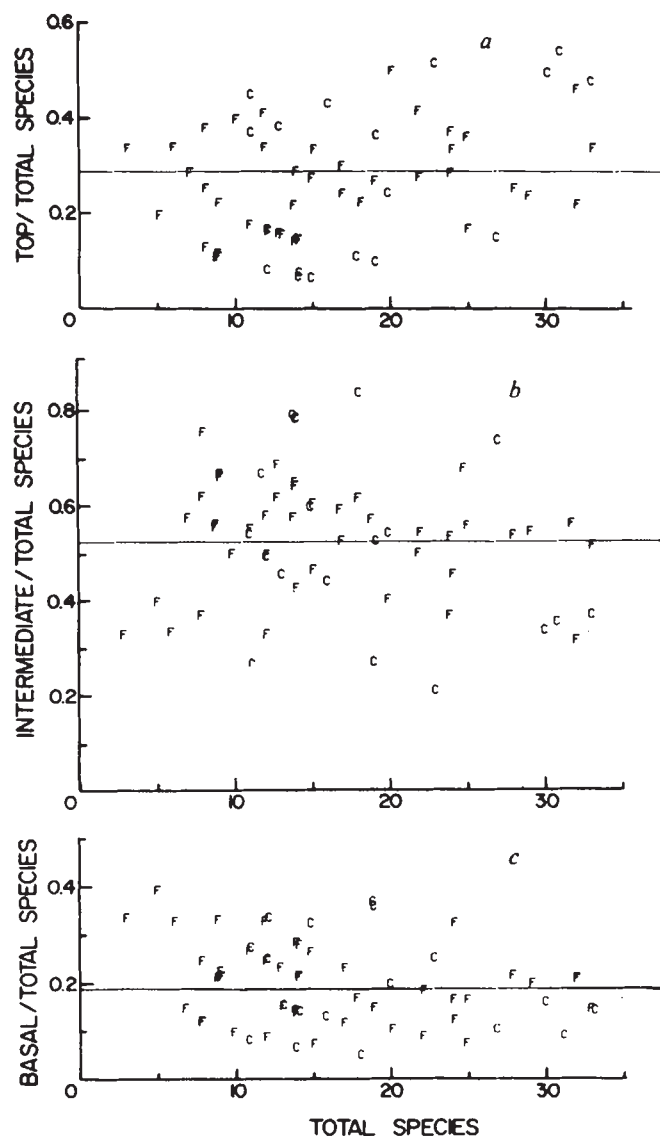


Fig. 2 Three ratios plotted as a function of the number of species. The fitted lines are constrained to be horizontal (slope = 0). *a*, Top species/total species. The height of the line is 0.2853. *b*, Intermediate species/total species. The height of the line is 0.5251. *c*, Basal species/total species. The height of the line is 0.1896.

y-axes share a similar quantity, namely the intermediate species, which are both prey and predators. What is surprising is the tightness of the fit, considering the size and heterogeneity of the sample examined. This suggests two possibilities: either the redundant variable, that is, the number of intermediate species, is very large compared with the number of basal and top species in most communities, or the proportions of all species in a food web that fall into each of these three categories are, overall, independent of the total number of species.

Figure 2 illustrates the reality of the second alternative: in the 62 webs examined, the fractions of top, intermediate and basal species are, on average, independent of the total number of species, although there is a slight tendency for the fraction of top species to increase and for the fraction of basal species to decrease as the total number of species increases. To obtain a global estimate of the proportions of species in each of the three categories (top, intermediate, basal), we summed over all food webs the observed numbers in each category, and divided by the sum total of species over all food webs. The global proportions of top, intermediate and basal species correspond to the heights of the horizontal lines in Fig. 2*a-c*.

The scatter of points about the horizontal lines in Fig. 2, when constant and fluctuating food webs are considered together, agrees with the hypothesis that in each food web the top, intermediate and basal species are multinomially sampled from the total species in proportions that are constant for all webs. If the species counts are arranged in a 3×62 contingency table with rows for top, intermediate and basal species and one column for each web, a homogeneity test yields a χ^2 of 138.9 with 122 d.f., which is not significant at the 0.1 level.

There is no evidence for a difference between constant and fluctuating food webs in the mean proportions of top, intermediate and basal species. A homogeneity test of a 3×2 contingency table with rows for top, intermediate and basal species and columns for constant and fluctuating food webs, and the summed species counts as cell entries, gives a χ^2 statistic of 1.4 with 2 d.f., which is not significant at the 0.1 level.

However, the proportions of top, intermediate and basal species in constant food webs, considered separately, are significantly more variable, and the proportions in fluctuating food webs, considered separately, are significantly less variable, than expected from multinomial sampling (using a 0.02 significance level). Separate homogeneity tests of the constant webs (in a 3×19 contingency table) and of the fluctuating webs (in a 3×43 contingency table) yield χ^2 of 82.8 with 36 d.f. and 56 with 84 d.f., respectively. The visual counterpart of this statistical result is the appearance in each panel of Fig. 2 of fluctuating food webs near the horizontal line and of constant food webs scattered above and below the band of fluctuating webs. Food web structure appears more constrained in fluctuating than in constant environments, as previously noted^{3,8}.

We now show that the empirical regularities in Fig. 1*b* can be derived from the approximate scale-invariance shown in Fig. 2.

Let S be the total number of species in a single community food web, T the expected number of top species in that web, I the expected number of intermediate species, B the expected number of basal species, R the expected number of predators and Y the expected number of prey. By definition

$$S = T + I + B \quad (1)$$

$$R = T + I \quad (2)$$

$$Y = I + B \quad (3)$$

By observation

$$T/S = p \quad \text{or} \quad T = pS, \quad \hat{p} = 0.2853 \text{ (Fig. 2a)} \quad (4)$$

$$I/S = q \quad \text{or} \quad I = qS, \quad \hat{q} = 0.5251 \text{ (Fig. 2b)} \quad (5)$$

$$B/S = r \quad \text{or} \quad B = rS, \quad \hat{r} = 0.1896 \text{ (Fig. 2c)} \quad (6)$$

Adding equations (5) and (6) and dividing by the sum of equations (4) and (5), we recover the observed regularity (Fig. 1*b*)

$$Y/R = a \quad \text{or} \quad Y = aR, \quad a = (q + r)/(q + p) \quad (7)$$

The predicted value $(\hat{q} + \hat{r})/(\hat{q} + \hat{p}) = 0.8819$ is identical to the observed $\hat{a} = 0.8819$ because of the formulas we used to estimate the slope a and the proportions p , q and r . However, the observation in Fig. 1*b* that the average number of prey is a linear function of the number of predators is not a tautologous consequence of the estimation formulas. The proportionality of prey to predators follows from the scale-invariance we have discovered here. Were data available, it would be interesting to examine whether the distribution of biomass into top, intermediate and basal species is also scale-invariant.

We conclude that the values of any two of the three parameters p , q and r summarize succinctly a substantial amount of information about the empirical regularities found in community food webs and provide a factually grounded benchmark against which the deviations of particular food webs may be measured. Why these proportions take the values they do and why the proportions are scale-invariant remain open questions.

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Monoclonal antibody that identifies subsets of neurones in the central visual system of monkey and cat

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Striking correlations between structure and function are found in the visual cortex of Old World primates^{1–4}. These include the co-localization of glutamic acid decarboxylase (GAD), the biosynthetic enzyme of the inhibitory neurotransmitter, γ -aminobutyric acid) with the mitochondrial enzyme, cytochrome oxidase (CO)⁵ in functionally distinct subcompartments^{6,7} of ocular dominance columns^{3,4,8}. We report here immunocytochemical studies with a monoclonal antibody (CAT 301)⁹ showing that the antibody recognizes an uncharacterized antigen on surfaces of some neurones in certain layers of the monkey striate cortex (area 17), and in certain parts of the cat and monkey dorsal lateral geniculate nuclei (LGN). Patches of immunocytochemically stained neurones and neuropil, apparent in layers III, IVB and VI of the striate cortex of normal monkeys, become even more clearly delineated in animals from which one eye has been removed. The antibody-stained patches in the three layers line up radially with one another in lines passing through the centres of ocular dominance columns (demonstrable by CO staining in layers IVA and IVC). In layers III and VI the patches coexist with CO-positive patches^{3,4} and, in the horizontal dimension, both antibody and CO-positive patches are aligned to form rows. Stained neurones in the monkey LGN are primarily in the magnocellular layers and in the cat LGN are confined to laminae A and A₁, the inter-laminar plexuses, the perigeniculate nucleus and the medial inter-laminar nucleus. The antigen we have localized is associated with particular cell populations, some of which may correspond to a specific, physiological class.

Three normal cynomolgus monkeys (*Macaca fascicularis*) and three cats were used. Three other monkeys were anaesthetized and one eye removed 8 or 10 days before they were re-anaesthetized and perfused with buffered 4% paraformaldehyde. Alternating frozen sections from both sides of the brains were processed immunocytochemically with CAT 301 (refs 9, 10) and histochemically for cytochrome oxidase¹¹.

CAT 301 stains pyramidal and nonpyramidal neurones particularly in layers IVB and VI of monkey striate cortex (Fig. 1); there is a diffuse staining of the neuropil around the stained cells (Fig. 1a). Some cells in layers II and III but very few in layers I, IVA, IVC or V are stained.

Within layers IVB and VI and deep in layer III, CAT 301-positive neurones form patches (Figs 2a,c, 3a). Patches in layers

III and IVB are 200 $\mu\text{m} \times 150 \mu\text{m}$; those in layer VI approach 300 μm . In tangential sections (Fig. 2) patches in layers IVB and VI form rows perpendicular to the border of areas 17 and 18. Gaps between patches vary in size from 300–500 μm along the rows to 200–400 μm between the rows (Fig. 2). Rows of patches are less distinct in layer III (Fig. 3).

In transverse sections most patches of CAT 301-positive cells in layer VI line up radially with patches in layers IVB and III (see Fig. 2). Less than 10% of those in layer VI are without overlying patches in layer IVB, but approximately 30% in layers VI and IVB have no corresponding patch in layer III.

Following the removal of one eye, the patchy distribution of CAT 301-positive cells in layer VI becomes much more distinct due to a sharp reduction in the neuropil staining at the edges of the patches.

Ocular dominance columns in area 17 are demonstrated by CO histochemistry after monocular enucleation (Fig. 2b,d). The columns are most obvious in layers IVA and IVC^{2,12} but they can also be distinguished in layers III and VI.

By aligning radially oriented blood vessels, tangential sections in layer IVC reacted for CO can be superimposed on adjoining sections stained immunocytochemically with CAT 301. Patches of CAT 301-positive cells in layers III, IVB and VI are aligned with the centres of both CO-rich and CO-poor columns in layers IVA and IVC (Figs 2a–c), indicating that CAT 301-positive patches lie at the centres of both ipsilateral and contralateral ocular dominance columns. Where the stripes formed by ocular dominance columns branch, two side-by-side patches of CAT 301-positive cells appear in layers IVB and VI. Patches are also present where the monocular segment of the visual field is represented and ocular dominance columns are not demonstrable^{2,12}.

In layer III, 70% of the CO-positive periodicities of layer III of normal monkeys^{3,4,7,8} (Fig. 3a) superimpose with CAT 301-positive patches (Fig. 3b). The remaining 30% are without corresponding CAT 301 patches. However, virtually all CO-positive periodicities in layer III line up radially with CAT 301 patches in layers IVB and VI.

In layer VI, with very few exceptions, patches of CAT 301-stained cells and patches of CO staining in layer VI are exactly superimposed (Fig. 2). Both lie at the centres of right- and left-eye dominance columns (Fig. 2c,d) and both are aligned in stripes.

In the LGN of enucleated and intact monkeys most cells (~95%) in the magnocellular layers and very few (<15%) in the parvocellular layers are CAT 301-positive (Fig. 4a). Stained cells vary from 18 to 24 μm in average diameter.

In the cat LGN, CAT 301-positive cells make up slightly more than 20% of the population of the A and A₁ laminae and only 1 or 2% of the population of the C laminae. Stained cells average 20–25 μm in diameter. Most neurones in the inter-laminar zones, the perigeniculate nucleus and the ventral lateral geniculate nucleus, and approximately 80% of those in the medial interlaminar nucleus are labelled (Fig. 4b).

The striate cortex of Old World monkeys can be divided into a series of functional modules or 'hypercolumns'^{11,12}. CO histochemistry has revealed a series of radially organized periodicities in layers II–III and V–VI^{3,4} that occupy the centres of ocular dominance columns. Several reports, focused on the periodicities in layer III, document their structural^{3,4,8} functional^{6,7,10,13,14} and biochemical^{5,15} properties and Livingstone and Hubel⁴ have pointed out that they may be an indication that hypercolumns are structural entities with definite borders. We have demonstrated that neurones in monkey striate cortex stained with CAT 301, are also organized in radial arrays that align with the CO-rich periodicities. Like the CO-positive zones, CAT 301-positive patches occupy the centres of ocular dominance columns, further demonstrating that groups of neurones within the columns possess distinct properties.

CAT 301-positive patches in area 17 superficially resemble patches of cells described as giving rise to long intracortical axons¹⁶ but, unlike CAT 301-positive patches, the long-axoned

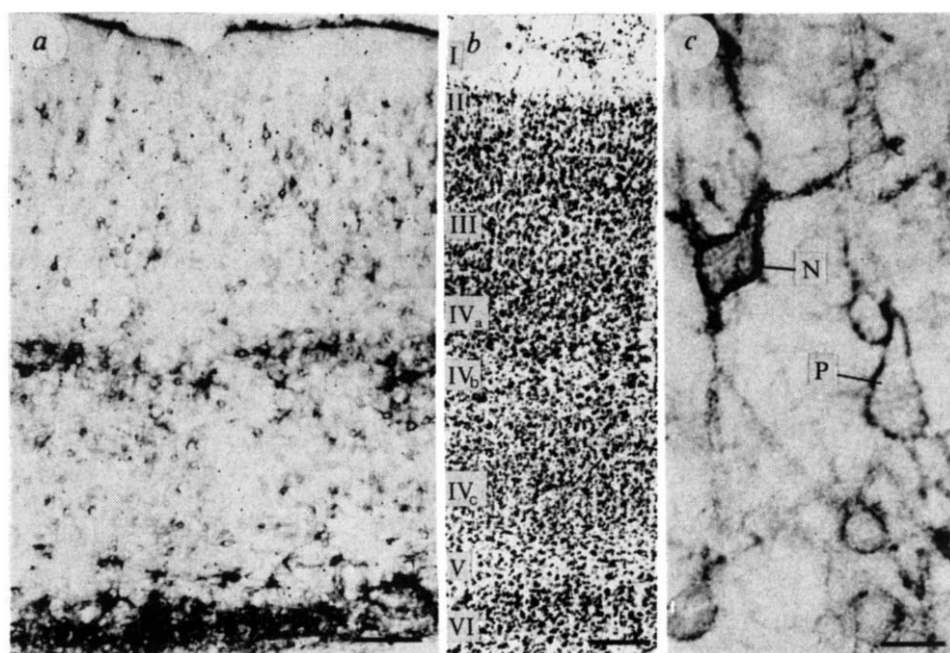


Fig. 1 *a*, Laminar distribution of CAT 301-positive neurones in a radial section through area 17 of a normal monkey. Dense bands of cells are stained in layers IVB and VI but very few cells are stained in other layers. *b*, Section adjacent to *a*, counterstained with thionin. Roman numerals indicate cortical laminae according to the scheme of Brodmann²⁶ as modified by Lund²⁷. *c*, Higher magnification of CAT 301-positive cells in layer VI of same section as *a*. Both pyramidal (P) and non-pyramidal types are stained. Scale bars, 100 μ m in *a* and *b*, 20 μ m in *c*.

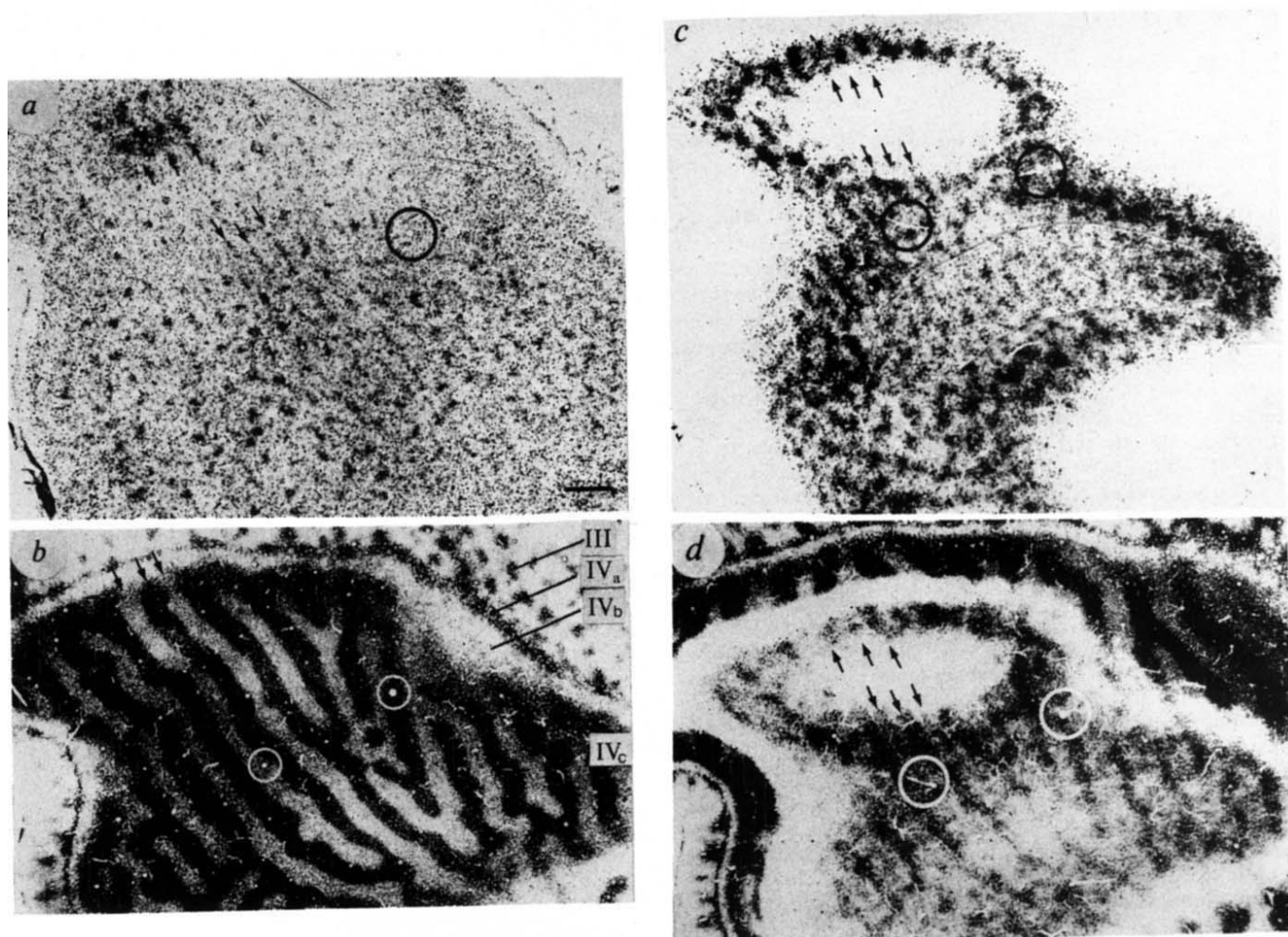


Fig. 2 Tangential sections (parallel to the brain surface) through area 17 of a monkey from which the contralateral eye was removed. *a*, Stained immunocytochemically by CAT 301, mainly through layer IVB; *b*, stained for CO, mainly through layer IVC. Alternating CO-rich and -poor bands represent ocular dominance stripes of intact and removed eyes, respectively. *c*, *d*, Stained by CAT 301 and for CO, respectively, mainly through layer VI. In *d* blanching of alternate rows of CO patches is an effect of the enucleation. Other layers indicated on *b* appear because plane of section is not perfectly parallel to the individual layers. The sections can be superimposed by aligning the penetrating blood vessels, two of which are circled. When this is done, the rows of CAT 301-stained periodicities can be seen to be in exact register with one another, with the ocular dominance stripes of layer IVC, and with the rows of CO-stained periodicities of layer VI. Three corresponding rows of patches and stripes are indicated by arrows. Note that the CO staining declines in ocular dominance stripes and patches related to the removed eye so that staining with CAT 301 is associated with both strongly and weakly CO-positive stripes and patches. Scale bar, 1 mm.

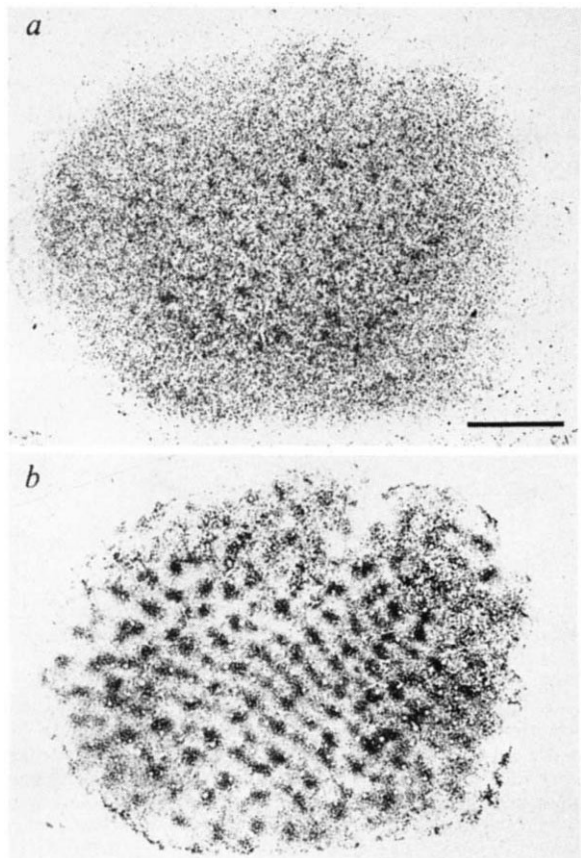


Fig. 3 Adjacent tangential sections through layer III in area 17 of a normal monkey. Patches of neurones stained immunocytochemically by CAT 301 (*a*) are aligned in rows that correspond to rows of cytochrome oxidase stained patches in the adjacent section (*b*). Many of the CAT 301-positive patches can be exactly superimposed on the CO positive densities. Scale bar, 2 mm.

cell patches are not correlated with CO-positive periodicities.

CO staining in layers III, IV and VI correlates with the termination of thalamic afferents⁴. CAT 301 patches coincide with CO staining in layers III and VI, but not in layers IVA or IVC where the heaviest thalamic terminations occur. CAT 301 is unlikely to mark afferent terminals directly because immunoelectron microscopy suggests that the surface antigen is extrasynaptic and synthesized by the cell which it outlines¹⁰, that is, the postsynaptic cell is defined by the antigen.

The distribution of CAT 301-positive neurones in monkey visual cortex and in monkey and cat LGN may reflect the distribution of neurones responding to visual stimuli of many wavelengths (broad band cells). These are localized in the magnocellular layers of the monkey LGN¹⁷⁻¹⁹ and in the A and A1 laminae and medial interlaminar nucleus^{20,21} of the cat LGN regions stained by CAT 301. In the monkey visual cortex broad band cells⁴ and CAT 301-positive cells are found in the CO-rich periodicities of layer III. Broad band cells may also be present in CO-rich bands of monkey area 18, which also coincide with CAT 301-positive patches (unpublished observations). CAT 301-positive cells are certainly not restricted to laminae in the LGN nor to layers in area 17 where broad band responses have been recorded, so their distributions in the visual system may be associated not only with broad band cells but also with other as yet unidentified cell colonies as well.

Previous studies with monoclonal antibodies to uncharacterized antigens have focused on classes of neurones identified by

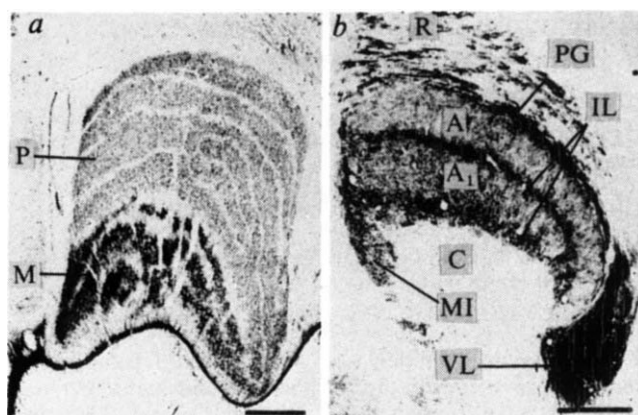


Fig. 4 Frontal sections of LGN from a monkey (*a*), and a cat (*b*), stained by CAT 301. *a*, In the monkey LGN there are many densely stained cells in the two magnocellular layers (M) and very few in the four parvocellular layers (P). The diffuse background staining of the parvocellular layers is not clearly associated with any neuronal processes. *b*, In the cat lateral geniculate complex, there is heavy staining of cells in the medial interlaminar nucleus (MI), the ventral lateral geniculate nucleus (VL), the perigeniculate nucleus (PG) and in the interlaminar plexuses (IL), with less staining of the A and A1 laminae and very little of the C laminae. Additional staining appears in the thalamic reticular nucleus (R). Scale bar, 1 mm.

their morphology, connectivity and/or position in an organized structure²²⁻²⁴. The present and previous^{9,10,25} reports demonstrate that CAT 301 recognizes surface molecules on many but not all neurones, not even those (for example, pyramidal cells) of the same morphological type. The nature of the neuronal surface molecule recognized by CAT 301 remains to be determined, as does the role of the molecule in the structure, function and development of the neurones it marks.

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Muscarinic inhibitory transmission in mammalian sympathetic ganglia mediated by increased potassium conductance

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Slow muscarinic inhibition may be a powerful influence on membrane properties in the peripheral¹⁻³ and central nervous system⁴. But the location of the muscarinic receptors in sympathetic ganglia, either on interneurons or on the postganglionic membrane, and the underlying mechanism of the inhibitory response, remains controversial⁵⁻⁷. In mammalian sympathetic ganglia synaptic activation of muscarinic receptors located on inhibitory interneurons was thought to release catecholamines leading to a membrane hyperpolarization called the slow inhibitory postsynaptic potential, or s.-i.p.s.p.⁸. However, the s.-i.p.s.p. in parasympathetic ganglia^{3,9,10} and in amphibian sympathetic ganglia^{1,2} is due to direct monosynaptic activation of muscarinic receptors, accompanied by an increased potassium conductance (but see ref. 11), and is not mediated by catecholamines. The situation is less clear in mammalian sympathetic ganglia and monosynaptic s.-i.p.s.p.s observed in other ganglia could be exceptions to the hypothesis. We showed earlier that the s.-i.p.s.p. in rabbit superior cervical ganglia is not affected by catecholamine antagonists¹². We now show that the s.-i.p.s.p. in a mammalian sympathetic ganglion is due to the monosynaptic activation of muscarinic receptors, probably by an increase in potassium conductance.

The superior cervical ganglia along with their pre- and postganglionic nerves were isolated from adult male New Zealand rabbits and prepared as described previously¹². Glass microelectrodes (30–100 MΩ) filled with 3 M KCl were used for intracellular recording from over 150 sympathetic neurones.

In all experiments, nicotinic responses were blocked by the administration of hexamethonium (500 μM) to facilitate examination of the s.-i.p.s.p. Iontophoretic application of acetylcholine (ACh, 2 M; 50–100 MΩ) elicited a hyperpolarization that mimicked the nerve-evoked s.-i.p.s.p. (40 Hz for 250 ms). Both responses were abolished by atropine (0.1 μM) but unaffected by the adrenergic antagonist phentolamine (1 μM) (Fig. 1). We previously showed with extracellular recordings that this concentration of phentolamine blocked hyperpolarizations induced by noradrenaline, adrenaline and

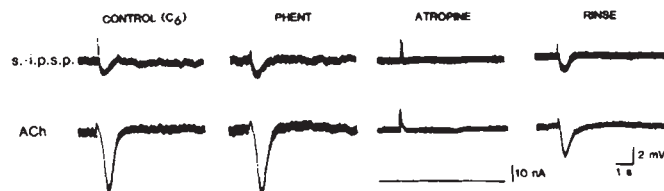


Fig. 1 Effect of antagonists on the nerve-evoked s.-i.p.s.p. and ACh hyperpolarization. Hexamethonium (C_6 , 500 μM) eliminated all fast nicotinic responses leaving only the slow muscarinic responses. The s.-i.p.s.p. was elicited by orthodromic stimulation at 40 Hz for 250 ms; in the same cell, a single iontophoretic pulse of ACh was applied (50 nA, 40 ms). Phentolamine (1 μM) had no effect on either the s.-i.p.s.p. or ACh hyperpolarization, even after 30-min application. However, the muscarinic antagonist, atropine (0.1 μM) completely eliminated both responses. The responses returned after rinsing for 1 h. A representative current trace is positioned below the third ACh record. Membrane potential –62 mV.

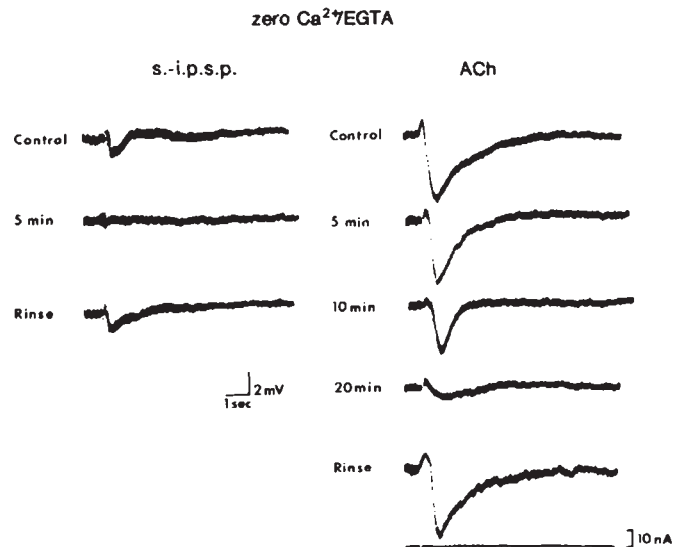


Fig. 2 Effect of blockade of synaptic transmission with 0 Ca^{2+} /EGTA solution on the nerve-evoked s.-i.p.s.p. and ACh hyperpolarization. The Ca^{2+} -free, high Mg^{2+} solution was prepared by substituting 2.5 mM $MgCl_2$ for $CaCl_2$ (3.7 mM $MgCl_2$, total); EGTA (1 mM) was also added. Both responses were recorded simultaneously from the same cell. Stimulation parameters are the same as in Fig. 1. The left-hand column shows the control s.-i.p.s.p., rapid block of the s.-i.p.s.p. in 0 Ca^{2+} solution and return of the response after rinse. The right-hand column shows that the simultaneously recorded ACh hyperpolarization was unchanged at the time when synaptic transmission was blocked (5 min). Prolonged exposure to the zero Ca^{2+} solution (20 min) eventually depressed the ACh response, possibly via a postsynaptic effect. Rinse records for both responses were recorded 20 min after return to control solution (10 and 20 min records in 0 Ca^{2+} media were omitted for the s.-i.p.s.p. as they were identical to the 5 min record). Membrane potential –60 mV.

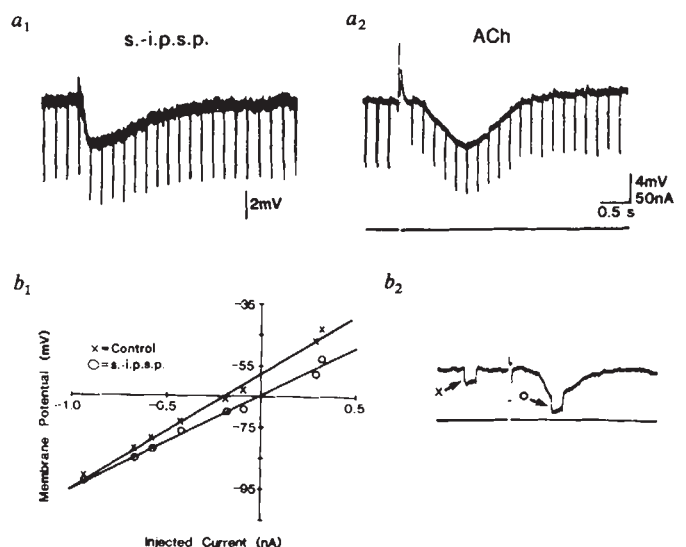
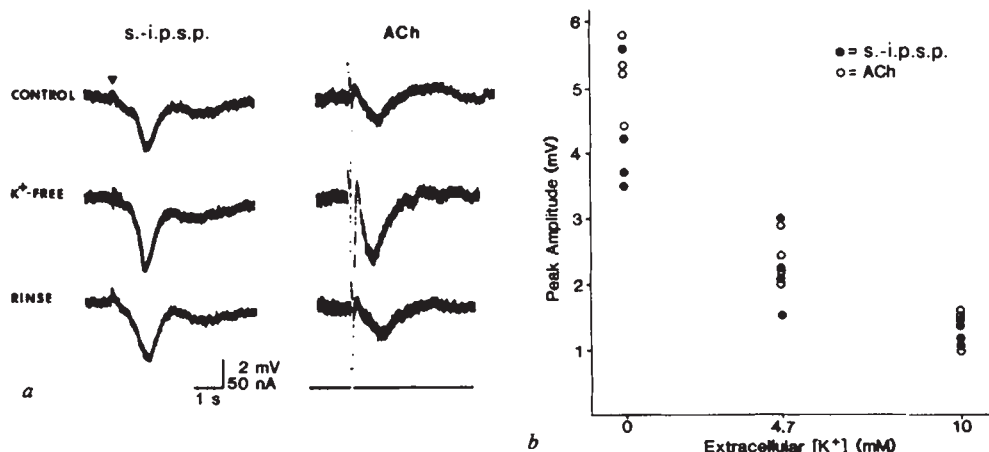


Fig. 3 Conductance changes during the s.-i.p.s.p. and ACh hyperpolarization (50 nA, 100 ms). *a*, Both responses were associated with a decrease in input resistance, reflected in the decrease in the amplitude of the anodal constant current pulses. Current trace is located below the voltage trace for the ACh response. Time calibration is the same for both records. Membrane potential –55 mV. *b*, Current-voltage plot for the s.-i.p.s.p. Hyperpolarizing and depolarizing current pulses of constant duration (300 ms, see *b*₂) and varying amplitude were passed across the membrane before (x) and at the peak of the s.-i.p.s.p. (o). The difference in the slope of the two plots indicates a decrease in resistance during the s.-i.p.s.p. The intersection point of the lines represents the estimated reversal potential of the response, here, –94 mV. Membrane potential –58 mV.

Fig. 4 Effect of extracellular K^+ concentration on the s.-i.p.s.p. and ACh hyperpolarization. **a**, Both the nerve-evoked s.-i.p.s.p. and hyperpolarization to a single iontophoretic pulse of ACh (40 nA, 100 ms) increased in amplitude when perfused with a K^+ -free solution. Equimolar concentrations of NaCl were substituted for KCl in the physiological solution. Membrane potential -60 mV. **b** Illustrates the change in peak amplitude of s.-i.p.s.p. ($\times$) and ACh hyperpolarization ($\circ$) versus extracellular K^+ concentration. Changing the K^+ concentration had no effect on the resting membrane potential of these cells.



dopamine¹². These data suggested that catecholamines do not mediate the s.-i.p.s.p. in mammalian sympathetic ganglia.

To investigate whether the s.-i.p.s.p.s are due to a disynaptic process, we tested the effect of blocking synaptic transmission on the response to iontophoretically applied ACh. Exposure to a 0 calcium/3.7 mM magnesium, EGTA (1 mM) solution quickly abolished the nerve-evoked s.-i.p.s.p. ($n = 3$, Fig. 2). In contrast, the ACh response persisted at a time when synaptic transmission was blocked, and was depressed only after prolonged exposure to the calcium-free solution (15 min) ($n = 8$). All responses returned to control levels during the rinse. Identical results were seen when synaptic release was blocked with tetrodotoxin (TTX, 1 μ M; $n = 4$). After 3 min of superfusion with TTX, the s.-i.p.s.p. was eliminated while in the same four cells the ACh response was not affected. These data do not support the disynaptic hypothesis. In every case the ACh hyperpolarization persisted when synaptic transmission (that is, the s.-i.p.s.p.) was blocked. This suggests that the muscarinic action of ACh is a direct effect on the ganglion cells and the calcium-free solution may be affecting underlying ionic mechanisms at the postsynaptic membrane. This would explain the persistence and gradual depression of the ACh iontophoretic response long after transmitter release had been eliminated.

We examined the mechanism underlying the s.-i.p.s.p. and ACh hyperpolarization by passing constant current anodal pulses through the recording electrode during the responses to analyse the conductance changes (Fig. 3). In cells where both the s.-i.p.s.p. and ACh response were recorded at the same membrane potential ($n = 12$) (Fig. 3a₁ and a₂) a small but consistent decrease in resistance was observed. These results reflect a similar increase in conductance during both the nerve-evoked s.-i.p.s.p. and the ACh hyperpolarization. In addition, current-voltage (I - V) plots were constructed by passing individual short duration current pulses across the membrane before and at the peak of the responses (Fig. 3b₂). A series of anodal and cathodal current pulses of constant duration (200 ms) and varying amplitude (-1.5 to $+1.0$ nA) were passed through the recording electrode just before and at the peak of the hyperpolarization. Curves were plotted of current against absolute membrane potential. Typical plots for control resistance values (pre-response) and values at the peak of the s.-i.p.s.p. are shown in Fig. 3b₁. A plot of the control resistance values against those values at the peak of the ACh hyperpolarization yielded similar results. The reversal potential estimated from the intersection of the current-voltage plots in the linear, non-rectifying part of the membrane¹³ was 102.7 ± 10 ($n = 4$) for the s.-i.p.s.p. and 100.4 ± 11 ($n = 5$) for the ACh hyperpolarization. These experiments implied that potassium and/or chloride ions may be involved in the generation of the s.-i.p.s.p. Since the hyperpolarizations were not reversed when KCl electrodes were used, it is unlikely that chloride ions are responsible for the responses. From our evidence with zero calcium solutions, it appears that calcium ions may also be involved, although more conclusive evidence is necessary.

We compared the effects of changing the external potassium ion concentration on the ACh hyperpolarization and the s.-i.p.s.p. As shown in Fig. 4, removal of potassium from the superfusing solution increased the amplitude of both hyperpolarizing responses ($n = 9$). The response to iontophoretic ACh was increased $40 \pm 2\%$ and the s.-i.p.s.p. was increased $58 \pm 19\%$. There was no change in the duration of either potential. When the extracellular potassium concentration was elevated to 10 mM, both the ACh and s.-i.p.s.p. hyperpolarizations were depressed. The hyperpolarization to iontophoretically applied ACh decreased $25 \pm 2\%$ and the s.-i.p.s.p. by $39 \pm 4\%$. Figure 4b summarizes the changes in the s.-i.p.s.p. and ACh hyperpolarization as the extracellular potassium concentration was changed. This agrees with the hypothesis that the s.-i.p.s.p. and the ACh hyperpolarization which mimics it, are generated by an increased membrane conductance to potassium ions.

We report here the first evidence in a mammalian sympathetic ganglion that the s.-i.p.s.p. is mediated by an increased potassium conductance via monosynaptic activation of muscarinic receptors. Furthermore, the potassium current may be a calcium-activated potassium conductance, dependent on an influx of calcium ions, as suggested by the effect of zero calcium solution on both responses. Calcium-activated potassium conductances in a variety of nerve and muscle cells have been shown to mediate long-lasting hyperpolarizations following calcium influx¹⁴⁻¹⁸. In addition, calcium-activated potassium conductances have been linked to muscarinic responses in a number of neuronal systems^{19,20}. These experiments provide further insight into the mechanisms of the muscarinic s.-i.p.s.p., a potential that may exert a powerful influence on membrane excitability and have an important role in the integration of autonomic function.

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Specific depletion of immunoreactive growth hormone-releasing factor by monosodium glutamate in rat median eminence

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A potent and specific growth hormone-releasing factor (GRF) was recently isolated and characterized from a human islet cell tumour of the pancreas that caused acromegaly¹⁻³. Antibodies raised against the synthetic replicate of this peptide have allowed the immunohistochemical identification of GRF-producing neurones within the primate central nervous system⁴. Such neurones are found mainly in the arcuate nucleus⁴⁻⁶ in human and monkey hypothalamus, suggesting that this nucleus is a primary source of GRF. We have further investigated this hypothesis by studying the anatomical organization of GRF neurones in rat hypothalamus, using an antibody raised against the recently characterized rat hypothalamic GRF⁷ in normal animals and in animals neonatally treated with monosodium glutamate (MSG), a treatment which results in the selective destruction of arcuate nucleus neurones⁸⁻¹⁰. We present here the results which show that GRF-producing neurones are located mainly in the arcuate nucleus of rats. MSG treatment results in the complete loss of GRF-immunoreactive cell bodies within this nucleus and provokes a selective disappearance of GRF-immunoreactive fibres in the median eminence. These results show that the arcuate nucleus is the origin of the GRF-containing fibres that project to the median eminence and establish the MSG-treated rat as an *in vivo* model for studying growth hormone secretion in the absence of neurohumoral GRF.

Antisera against rat hypothalamic GRF were prepared by immunizing rabbits with a mixture of synthetic rat GRF and methylated bovine serum albumin emulsified in Freund's adjuvant as described previously¹¹. Immunohistological studies were performed on tissue sections from 19 male rats of 3 and 11 months of age. Nine animals were either untreated or injected with saline and 10 animals were injected with MSG (4 g per kg subcutaneous, s.c.) on alternate days for the first 10 days of life⁹. This dose is known to produce an 80-90% loss of neuronal perikarya in the arcuate nucleus⁸⁻¹⁰. To increase peptide content in cell bodies, some rats received a colchicine injection (50 µg in 50 µl saline) in the lateral ventricle 48 or 72 h before they were killed. Brains were fixed by intracardial perfusion of anaesthetized animals (chloral hydrate, 3 mg per kg) with chilled 4% paraformaldehyde in 0.12 M phosphate buffer pH 7.2. The brains were cut into blocks and rinsed in phosphate buffer with increasing concentrations of sucrose (12-16%). Frontal sections 10-16-µm thick were prepared as described⁴, mounted on gelatin-coated slides and stored at -20 °C until used. An indirect immunofluorescence technique was performed as described previously⁴; antibodies against rat GRF were used at a final dilution of 1:200 to 1:800. In addition, we used antibodies against ovine corticotropin-releasing factor (CRF), somatostatin and luteinizing hormone-releasing factor (LHRF) for comparative topographical studies. Controls for specificity of the immunostaining consisted of a comparison of adjacent sections treated with anti-GRF serum alone or with anti-GRF serum previously incubated with rat GRF (0.5 mg ml⁻¹ undiluted antibody) or PHI or vasoactive intestinal peptide (up to 10 mg ml⁻¹), two peptides having considerable structural homology to rat GRF⁷. Only preincubation with synthetic rat GRF resulted in the disappearance of specific immunostaining.

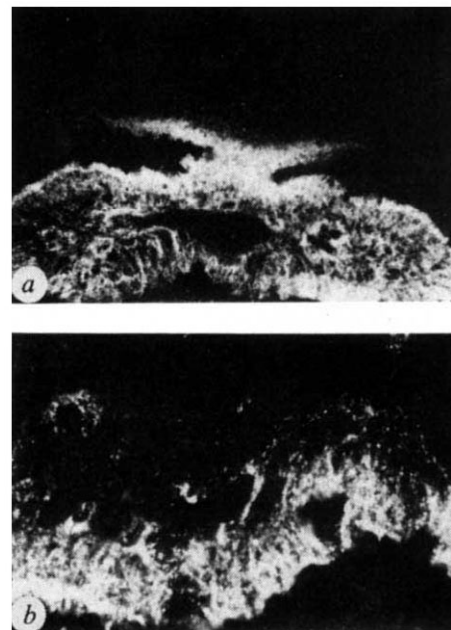


Fig. 1 Immunofluorescent staining of the median eminence of a normal adult rat with anti-rat GRF antiserum. *a*, General view of a frontal section of the posterior median eminence and the hypophyseal stalk, showing GRF-immunoreactive fibres in contact with the portal vessels. *b*, Higher magnification of a section stained with anti-rat GRF antiserum showing the course and termination of individual fibres.

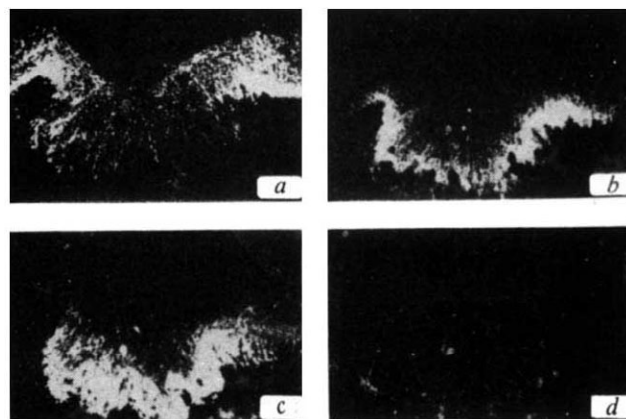


Fig. 2 Immunofluorescent staining of the median eminence of adult rats treated with monosodium glutamate (4 g per kg s.c.) on alternate days for the first 10 days of life. Immunoreactive fibres were observed in sections stained with anti-LHRF antiserum (*a*), anti-somatostatin antiserum (*b*) and anti-CRF antiserum (*c*) but were conspicuously absent from sections stained with anti-rat GRF antiserum (*d*).

In control rats, GRF immunoreactivity was constantly found in dense bundles of fibres in the median eminence, terminating in contact with portal vessels (Fig. 1), as well as in scattered fibres of the hypothalamus. No immunoreactive cell bodies were observed in animals not pretreated with colchicine. In rats pretreated with colchicine, numerous immunoreactive cells were found in the arcuate nucleus and the ventral and dorsolateral hypothalamus (Fig. 3), especially in the perifornical area and premammillary nuclei. Very few immunoreactive cell bodies were found in the ventromedial nucleus. In rats neonatally treated with MSG, the median eminence showed no GRF-immunoreactive fibres, while the distribution of LHRF-, somatostatin- and CRF-immunoreactive fibres was identical to that of control animals (Fig. 2). In the MSG-treated rats that received colchicine, GRF-immunoreactive cell bodies were only observed in the dorsolateral and ventral hypothalamus but not in the arcuate nucleus.

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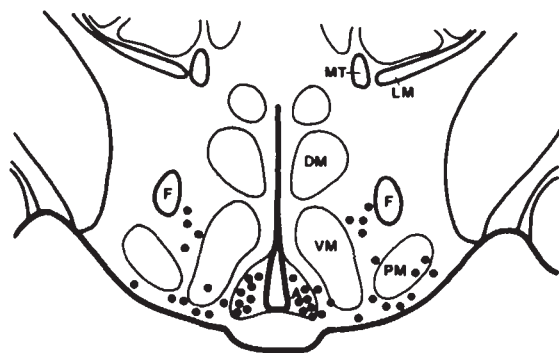


Fig. 3 Diagram of a frontal section of rat hypothalamus showing localization of GRF-immunoreactive cell bodies. A, arcuate nucleus; DM, dorsomedial nucleus; F, fornix; LM, medial lemniscus; MT, mamillothalamic tract; PM, ventral premamillary nucleus; VM, ventromedial nucleus.

Thus, the results obtained in normal rats show that the arcuate nucleus is the main source of GRF as has been found in man and monkey⁴⁻⁶. A very low abundance of neurones containing GRF immunoreactivity in the ventromedial nucleus of rats as well as primates suggests that this nucleus has a minor role in GRF production. This is in contrast to what has been hypothesized on the basis of electrical stimulation¹² and lesion experiments¹³. Since most of the GRF cell bodies are located in areas contiguous to the ventromedial nucleus, it is probable that the effects observed by destruction or stimulation of the ventromedial nucleus were due to extension of the lesion or diffusion of the stimulation to adjacent areas. Nevertheless, it cannot be excluded that neurones of the ventromedial nucleus might exert a direct stimulatory effect on GRF neuronal activity in the arcuate nucleus.

The disappearance of GRF immunoreactivity in the arcuate nucleus and median eminence after MSG treatment demonstrates that the arcuate nucleus is the source of the GRF fibres in the median eminence ending in contact with portal vessels. These results correlate well with the dramatic decrease of GRF bioactivity in the median eminence of MSG-treated rats reported by Acs *et al.*¹⁴. Actual quantification of this decrease must await the development of a radioimmunoassay for rat GRF. Although MSG treatment has been reported to result in a decrease in total pituitary growth hormone content in rats^{15,16}, pituitaries from such animals seem to have normal growth hormone secretion *in vitro*¹⁷ and have a normal response to GRF *in vivo*¹⁸. Thus, the dramatic impairment of growth observed in MSG-treated rats must be related to the selective destruction of the GRF neurones projecting in the median eminence, a conclusion supported by previous physiological studies¹⁵. The specificity of MSG in destroying GRF-containing neurones within the arcuate nucleus suggests that MSG-treated rats may serve as a useful *in vivo* model for the study of selective growth hormone deficiency of hypothalamic origin, an entity also observed in human neuroendocrinology¹⁹.

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Astrocytes present myelin basic protein to encephalitogenic T-cell lines

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Astrocyte proliferation and perivascular lymphocyte infiltration are conspicuous among the cellular changes in the active brain lesions of multiple sclerosis patients¹. Recent observations have indicated that most of the perivascular lymphocytes are T cells²⁻⁴ which may be actively involved in the generation of the brain lesions. Much less is known about the significance of the proliferative astrocytes, although the fact that they produce an interleukin-1 (IL-1)-like factor that enhances the release of interleukin-2 by T lymphocytes^{5,6}, may provide a clue. We show here that rat astrocytes are able to present antigen to T lymphocytes in a specific manner which is restricted by the major histocompatibility complex (MHC) and that they can in particular activate myelin basic protein (BP)-specific, encephalitogenic T-cell lines. Only on such interaction do astrocytes express Ia antigens in easily detectable amounts. Antigen presentation by astrocytes may have a central role in the generation of immune responses in the brain.

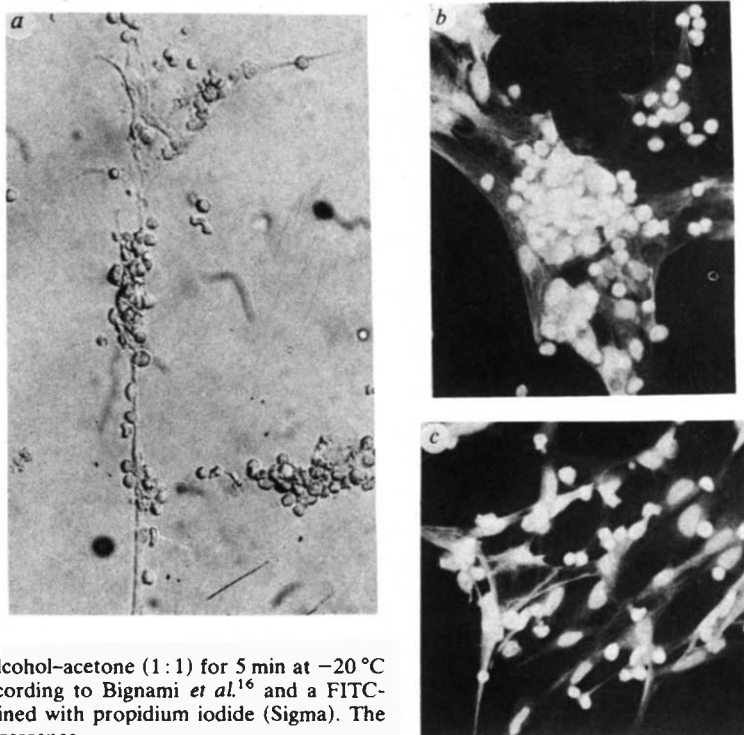
T lymphocytes cannot recognize free antigen but are dependent on antigen presentation by accessory cells. This function, which is MHC-restricted and requires IL-1, has been attributed to macrophages⁷⁻¹¹ and more recently to dendritic cells¹²⁻¹⁴. In several studies accessory cell function has been found to be mediated also by other Ia-positive cells such as Langerhans cells of the skin and endothelial cells¹⁵. Here we evaluate astrocytes for their ability to present antigen.

Immune cytochemical studies using rabbit antisera against glial fibrillary acidic protein (GFAP), a defined astrocyte marker¹⁶, revealed that astrocytes derived from newborn rats could be cultured to almost complete purity¹⁷. Such glial cell cultures were trypsinized after 3-4 weeks *in vitro*, and the cells plated on coverslips. Practically all of these cultured brain cells were GFAP-positive. In fact, if after trypsinization of glial cell cultures, the cells were plated on coverslips at a density of 2×10^5 cells, less than 20 cells per coverslip were found not to react with anti-GFAP antisera of three different sources (M. Schachner¹⁸, A. F.⁵ and Dakopatts Ltd). These results were considered to reflect the cell composition of the original cultures, as in non-confluent monolayers quantification of GFAP-negative cells was possible and during the staining procedure with anti-GFAP antibodies no loss of cells was observed. The cultures were also studied by indirect immunofluorescence using antibodies against galactocerebroside¹⁹, the major glycolipid in myelin, which is thought to be a specific cell-surface marker for oligodendrocytes^{20,21}. No galactocerebroside-positive cells were seen in our cultures. To exclude contamination by microglia cells/macrophages, the amount of cells with receptors for the Fc portion of immunoglobulin was determined using the techniques of Raff *et al.*²⁰. A small amount of positive cells with a vacuolar labelling pattern due to ingested fluorescein isothiocyanate (FITC)-conjugated immunoglobulin-anti-immunoglobulin complexes became detectable in primary

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Fig. 1 *a*, The attachment of BP-specific Lewis T cells to Lewis astrocytes in the presence of soluble BP. *b*, *c*, Astrocytes with large, oval nuclei and GFAP-positive processes and cytoplasmic filaments; in contrast, the nuclei of T cells appear smaller, round and intensely fluorescent. *b* Shows tight clusters of BP-specific Lewis T cells on Lewis astrocytes in the presence of BP. In control cultures of astrocytes of the MHC-congenic L.BN origin treated with BP, the T cells are randomly distributed and unblasted (*c*).

Methods: For astrocyte cultures, brains of newborn rats (Lewis, L.BN or (Lewis $\times$ BN) F_1) were dissociated according to the method of Manthorpe *et al.*¹⁷. After trypsinization, 10^6 brain cells were plated in 5 ml glial culture medium (Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and $300 \mu\text{g ml}^{-1}$ L-glutamine) in 25-cm^2 tissue culture flasks. Medium was changed on alternate days. After 3–4 weeks, the flasks were vigorously shaken by hand (see ref. 34), the detached cells discarded and the monolayer was washed three times. After this procedure the monolayer was trypsinized, the cells were reseeded on coverslips and allowed to adhere and spread for 2 days. Then BP- or OVA-specific Lewis cells and the corresponding antigen (BP or OVA) were added and cultured for 3–6 days. Direct interactions between T cells and astrocytes were studied by bright-field microscopy. To demonstrate the astrocyte-specific glial fibrillary acidic protein (GFAP), the cells on the glass slides were immersed in phosphate-buffered saline, dried, fixed in alcohol-acetone (1:1) for 5 min at -20°C and stained with rabbit antisera against GFAP prepared according to Bignami *et al.*¹⁶ and a FITC-conjugated goat anti-rabbit IgG. Cell nuclei were counterstained with propidium iodide (Sigma). The slides were examined for green (GFAP) and red (nuclei) fluorescence.



cultures but disappeared completely after passaging by trypsinization and reseeded of the astrocytes. The same picture emerged when testing a monoclonal antibody (W3/25) which stains both rat helper T cells and macrophages²². Cells of passaged astrocyte cultures did not label with anti-W3/25.

To test antigen presentation, graded amounts of passaged astrocytes were co-cultivated with cells of permanent BP-specific T-lymphocyte lines. These T-cell lines were originally derived from lymph node cells of rats immunized with BP²³. They are kept in long-term culture by repeated restimulation with BP in the presence of irradiated normal syngeneic thymus cells as accessory cells and by T-cell growth factor-dependent expansion between the restimulations. They express the 'helper' cell phenotype (W3/25+)²⁴. Within 24 h of culture in the presence of BP, BP-specific T cells of Lewis rat origin (L-BP) adhered tightly to the syngeneic astrocytes and formed aggregates of 10 and more lymphocytes per astrocyte (Fig. 1*a*). Within 48–72 h of culture, the adherent T cells transformed to large activated blast cells. This interaction was immune-specific: when ovalbumin (OVA) instead of BP was added to L-BP-astrocyte cultures, the T cells were randomly distributed, with no signs of physical contact with the astrocytes during observation periods of up to 72 h. Moreover, T-cell-astrocyte complexes were not formed when L-BP T cells were cultured in the presence of BP with MHC-congenic L.BN astrocytes. The popula-

tion of brain cells binding antigen-specific syngeneic T cells was identified as astrocytes by using GFAP as a marker (Fig. 1*b, c*). In either antigen or MHC-incompatible cell systems T lymphocytes remained in the state of small resting cells.

Morphological activation of astrocyte-associated T-cell lines resulted in their rapid proliferation. This was quantified by culturing T cells in the presence of both antigen and irradiated astrocytes and measuring ^3H -thymidine uptake. As a positive control, thymus was used as an optimal source of accessory cells. The results confirmed the microscopic observation. As shown in Fig. 2*a*, when L-BP T cells were cultured with astrocytes of Lewis rat origin in the presence of BP, ^3H -thymidine incorporation was highly elevated. The response was dependent on the number of astrocytes, with astrocyte-to-responder ratios lower than one. The response of L-BP T cells was dependent on the specific antigen BP, as no proliferation was observed when, as an irrelevant antigen, OVA was added to the T cells instead of BP (Fig. 2*a*). On the other hand, OVA was highly immunogenic to an OVA-specific Lewis T-cell line, when presented by Lewis astrocytes. The incorporation of ^3H -thymidine by 10^4 OVA-treated T cells was 33 ± 7 c.p.m. and in the presence of 10^4 irradiated syngeneic astrocytes $9,859 \pm 2,469$ c.p.m. These experiments demonstrate antigen specificity of astrocyte-dependent T-cell proliferation.

Antigen presentation by astrocytes was restricted by the MHC. In the presence of BP, significant incorporation of ^3H -thymidine by 10^4 responder L-BP T cells was produced by syngeneic Lewis rat astrocytes and astrocytes from (Lewis $\times$ BN) F_1 hybrids (Fig. 2*b*). No response was observed in the presence of MHC-congenic L.BN astrocytes. These L.BN astrocytes, however, functioned perfectly well as accessory cells when added to a BP-specific T-cell line from a (Lewis $\times$ BN) F_1 rat (data not shown).

There is ample evidence that Ia antigens expressed by antigen-presenting cells regulate immune responsiveness in concert with the Ia-restricted T-cell receptor²⁵. So far, Ia antigens have been found on cells having accessory function such as macrophages and dendritic cells. The MHC-restricted astrocyte-mediated activation of T helper lymphocytes would require the presence of Ia antigens on astrocytes also. It has been shown that the antigen recognition by our T-cell lines is only possible in the context of Ia antigen on the presenting cells, as evidenced by restriction experiments (E. Günther *et al.*, in preparation),

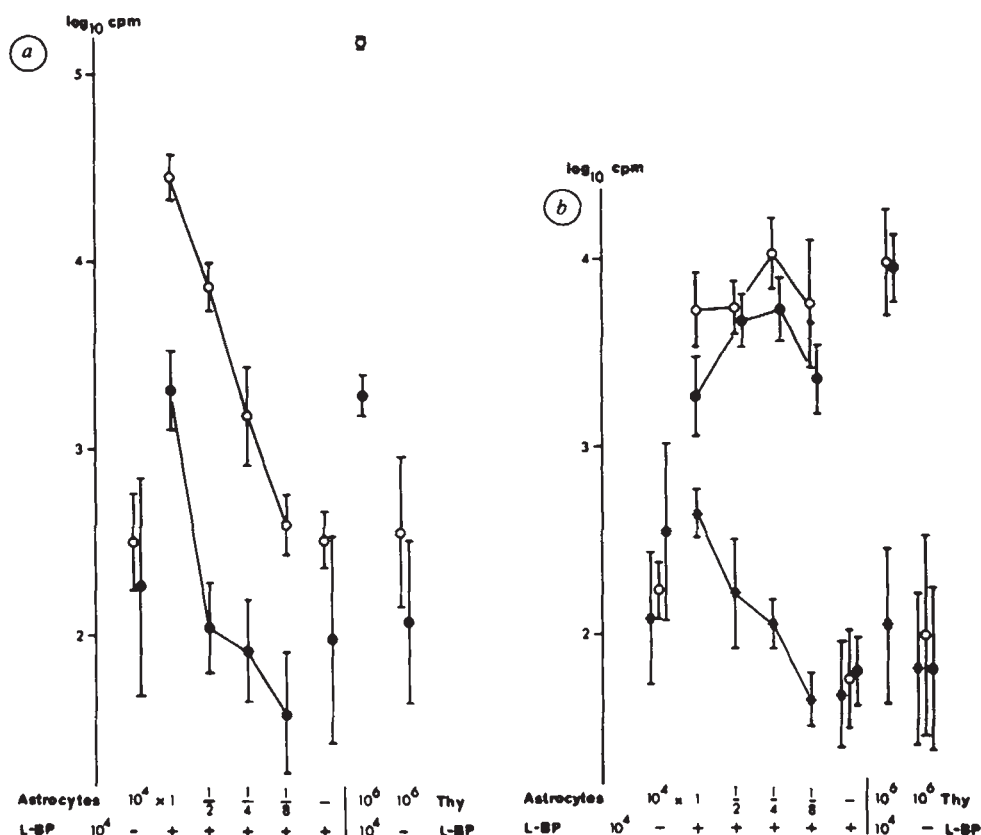
Table 1 Ia expression by astrocytes after specific interaction with T cells

Astrocyte treatment	Anti-Ia staining pattern of astrocytes of	
	Lewis rats	L.BN rats
Con A	—	—
L-BP + BP	OX6, OX3 HL	—
L-BP + Con A	OX6, OX3 HL	OX6

BP-specific cells of Lewis rat origin (L-BP) were co-cultured with astrocytes and BP or Con A ($5 \mu\text{g ml}^{-1}$). Astrocyte cultures were double stained with anti-Ia monoclonal antibodies and anti-GFAP antiserum as described in Fig. 3 legend. Two anti-Ia antisera were used, the OX6 being an anti-Ia common determinant and the OX3 HL an anti-polymorphic determinant (Lewis-specific)³³. —, No reactivity; OX6 or OX3 HL, positive staining of astrocytes with the anti-Ia antiserum OX6 or OX3 HL (Sera-Labs).

Fig. 2 *a*, L-BP T cells co-cultured with Lewis astrocytes or Lewis thymus cells in the presence of BP (○) or OVA (●). *b*, L-BP T cells added in the presence of BP to astrocytes or thymus cells of Lewis (○), L.BN (*) or (Lewis×BN)F₁ (●) origin. Data are given as log₁₀ c.p.m. of ³H-thymidine incorporation ± 95% confidence limits.

Methods: BP-specific Lewis T-cell lines (L-BP) were prepared according to the method of Ben-Nun *et al.*²³. Astrocytes were prepared as described in Fig. 1 legend, irradiated (3,000 rad) and co-cultured in quadruplicate in round-bottomed microtitre wells (0.2 ml) with L-BP T cells in the indicated concentrations. As a positive control, 10⁶ irradiated thymus cells were used instead of astrocytes. The medium consisted of DMEM supplemented with 1% Lewis rat serum L-glutamine (300 µg ml⁻¹), L-asparagine (36 µg ml⁻¹) and indomethacin (1 µg ml⁻¹). ³H-thymidine was added (0.2 µCi per well, specific activity 2 Ci mmol⁻¹) after 48 h of culture and cells were collected after another 14 h.



blocking experiments and adaptation of Ia from presenter cells by T-cell line during activation (H.W., in preparation). We have been unable to demonstrate Ia antigens on cultured naive astrocytes with anti-Ia antisera, but this does not exclude the possibility that a low concentration of functionally active Ia molecules is present. More important, however, we could demonstrate the expression of Ia antigens by astrocytes after specific contact with T lymphocytes (Table 1, Fig. 3). The induced Ia antigens were expressed by astrocytes as shown by double staining with anti-Ia and anti-GFAP antisera (Fig. 3) and by the phenotype of the expressed Ia following the genotype of the astrocytes rather than that of the lymphocytes. The allogeneic activation of the Lewis T-cell line L-BP by L.BN astrocytes and concanavalin A (Con A), which overrules MHC restriction, induced Ia antigens on GFAP-positive cells, which carried the non-Lewis phenotype (Table 1). The induction of Ia expression by astrocytes after specific contact with T lymphocytes, and perhaps also after other stimuli, could explain the controversial results of recent immune histological studies on Ia expression in mouse brain^{26,27}. On human glioblastoma cells binding assays clearly revealed HLA-DR determinants²⁸.

Other investigators have shown that an Ia-positive subpopulation of macrophages and/or the dendritic cells are most active in antigen presentation⁷⁻¹⁴. Contamination of astrocyte cultures with dendritic cells is unlikely, as, in contrast to astrocytes, the rat dendritic cell is a nonadherent cell. Furthermore, it has been demonstrated in the rat that dendritic cells are widely distributed in the interstitial connective tissues (for example spleen, liver, skin) but have not been found in brain. At least a proportion of GFAP-negative cells detected in our cultures (0.01%) could represent macrophages. Using different techniques it was found that the non-passaged primary brain cell cultures contained small amounts of macrophages, while contamination with macrophages was not observed in the passaged cultures used for the T-cell proliferation assay. However, trace amounts of undetected contaminating macrophages or dendritic cells could be a possible source of error in our experiments. We regard this theoretical possibility as very unlikely. Evidence supporting

the antigen-presenting function of astrocytes are both the finding of a MHC-restricted and antigen-specific physical contact observed between the astrocytes and the T cells, as well as the induction of Ia antigen expression by astrocytes exposed to activated T cells. In addition, previous studies showing the release of IL-1-like factors by mouse astrocytes stimulated with endotoxin⁵, rat C6 glioma cells⁶, rat primary astrocyte cultures (A.F., unpublished observation) and human glioblastoma cells²⁹ further substantiate the hypothesis that astrocytes are active accessory cells.

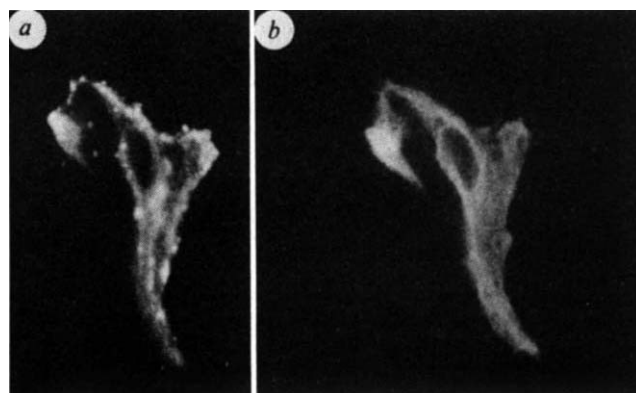


Fig. 3 To demonstrate Ia antigens, Lewis astrocytes were co-cultured with syngeneic BP-specific T cells and BP in round-bottomed microtitre wells as described in Fig. 2 legend. After 3 or 6 days of culture, cells were removed from the microtitre wells and stained with monoclonal anti-Ia antibodies³³ and rhodamine-conjugated rabbit anti-mouse immunoglobulin. Smears of stained cells were dried, fixed and stained for GFAP as described in Fig. 1 legend. The cells were examined for red fluorescence demonstrating cell-surface Ia antigens (*a*) and green fluorescence demonstrating intracellular GFAP (*b*). The relatively globular form of the astrocyte without long processes results from the incubation condition in the round-bottomed microtitre well where astrocytes in the cell pellet are prevented from adherence and spreading.

The role of astrocytes as accessory cells could be of primary importance in presenting cerebral antigens to infiltrating lymphocytes within inflammatory lesions of brains from patients with multiple sclerosis. This, and the finding that astrocytes themselves can be activated by factors released from stimulated lymphocytes³⁰, may connect two morphological hallmarks of the active multiple sclerosis lesion, that is, T-cell infiltration and astrocyte proliferation¹. More generally, astrocytes may have a previously unrecognized physiological cell function pivotal to the generation of immune responses within the brain tissue. The central nervous system has been considered to be an immunologically privileged site as it lacks a lymphatic drainage and is shielded from the blood by the blood-

brain barrier. At the site of this barrier an intense contact has been observed between astrocytes and the brain vascular tissue^{1,31}, and therefore an active contact between the immune system and the brain may directly depend on astrocyte function. Whether the accessory cell function of astrocytes is applicable to all or only a subpopulation of astrocytes such as process bearing type II cells (see ref. 32) remains to be determined.

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Hydroxyl radical scavengers inhibit human natural killer cell activity

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As natural killer (NK) cell activity is an essential constituent of host defence systems¹ and reactive oxygen intermediates participate in such defence²⁻⁴, the effect of scavengers of oxygen radicals on NK cell activity was investigated. Hydroxyl radical (OH[•]) scavengers⁵ (dimethyl sulphoxide (DMSO), thiourea, dimethylurea, tetramethylurea, benzoic acid, ethanol, methanol and ethylene glycol) inhibited NK cell activity. Catalase, a scavenger of H₂O₂, and superoxide dismutase (SOD), a scavenger of O₂⁻, either alone or in combination, did not inhibit NK cell activity. Inhibition of the lipoxygenase pathway of arachidonic acid metabolism, a potential source of cellular OH[•], with nordihydroguaiaretic acid⁶ and 5,8,11,14-eicosatetraynoic acid (ETYA)⁷ resulted in marked inhibition of NK cell activity. Inhibition of the cyclooxygenase pathway^{7,8} with acetylsalicylic acid or indomethacin had minimal effects on NK cell activity. Taken together, these findings suggest that OH[•], possibly generated via the lipoxygenase pathway of arachidonic acid metabolism, is critical for NK cell cytotoxicity.

NK cell activity is inhibited by a variety of structurally unrelated compounds that all share the property of being able to scavenge OH[•] (Fig. 1). Statistically significant inhibition of NK cell activity mediated by large granular lymphocytes (LGL), which are the principal NK cells in peripheral blood, with MOLT-4 as target cells is shown in Fig. 1a, and with K-562 as target cells in Fig. 1b. Urea, which is structurally related to thiourea, tetramethylurea and dimethylurea, but is not an OH[•] scavenger, did not inhibit NK cell activity even at 50 mM concentration. Other OH[•] scavengers, such as ethanol, methanol and ethylene glycol, also mediated inhibition of NK cell activity. The alcohols are relatively less efficient scavengers⁵ of OH[•] and

inhibition of NK cell activity required higher concentrations than for the other agents. Fifty per cent inhibition of NK cell activity was observed with LGL as effector cells and MOLT-4 or K-562 as target cells with the addition of methanol (315 mM), ethanol (80 mM) and ethylene glycol (320 mM) to the NK test system. None of the OH[•] scavengers affected the binding capacity of LGL to target cells at concentrations that resulted in significant inhibition of NK cell activity as tested in the target cell-binding assay⁹. The target cell-binding assay was performed by incubating LGL with MOLT-4 or K562 at an effector to target ratio of 1:1. LGL (1 × 10⁶ cells ml⁻¹) were incubated with target cells (1 × 10⁶ cells ml⁻¹) for 30 min at 4 °C with or without various OH[•] scavengers. The cells were then resuspended and a minimum of 200 effector cells were counted in a Neubaur Counting Chamber covered with an acridine orange-coated coverslip with a simultaneous UV light and phase contrast illumination to determine the number of effector-target conjugates. The use of acridine orange-coated coverslips and UV light¹⁰ allowed us to clearly distinguish between effector and target cells.

The addition of Me₂SO (75 mM), thiourea (10 mM), tetramethylurea (10 mM), dimethylurea (25 mM), benzoic acid (10 mM), ethylene glycol (320 mM), ethanol (80 mM) and methanol (315 mM) to the NK test system did not result in significant alterations in the number of effector-target conjugates formed. Twenty-five–40% of LGL formed conjugates with the target cells in the presence or absence of OH[•] scavengers.

Nonspecific toxicity of agents used as OH[•] scavengers may reasonably be excluded as an explanation for their inhibition of NK cell activity as target cells released less ⁵¹Cr in the presence of the OH[•] scavengers. Several additional experiments were performed to exclude further the possibility that nonspecific toxic effects of OH[•] scavengers were responsible for the inhibition. First, none of the OH[•] scavengers affected the viability of LGL, as determined by Trypan blue exclusion at concentrations that mediated significant inhibition of NK cell activity (Table 1, protocol 1). Second, LGL pretreated with DMSO (150 mM), thiourea (50 mM) and tetramethylurea (25 mM) and subsequently washed before being used as effector cells, exhibited normal NK cell activity (Table 1, protocol 2). The third and perhaps most compelling evidence against nonspecific toxicity

Table 1 Lack of nonspecific toxicity of OH⁻ scavengers

Protocol	OH ⁻ scavenger (mM)				
	DMSO (150)	Thiourea (50)	Ethylene glycol (320)	Methanol (630)	Tetramethylurea (25)
(1) Cell viability	100 (3)	100 (3)	100 (3)	100 (3)	100 (3)
(2) Pretreatment	130 ± 10 (4)	113 ± 9 (4)	ND	ND	125 ± 13 (4)
(3) Antibody-dependent cell-mediated cytotoxicity	103 ± 6 (6)	87 ± 9 (10)	81 ± 8 (3)	86 ± 5 (3)	ND

Protocol 1: Cell viability was determined using a Trypan blue exclusion assay. Per cent of viable cells found in three experiments is shown. **Protocol 2:** Large granular lymphocytes were incubated with OH⁻ scavengers for 3 h at 37 °C, washed three times with PBS, then used as effector cells with MOLT-4 as target cells. Results are expressed as per cent specific ⁵¹Cr release found with untreated LGL as effector cells. **Protocol 3:** Antibody-dependent cell-mediated cytotoxicity (ADCC) was determined with peripheral blood mononuclear cells as effector cells, and antibody-coated chicken red blood cells as target cells in a 3-h ⁵¹Cr release assay²³ (effector:target ratio is 5:1). Results are expressed as per cent specific ⁵¹Cr release found in OH⁻ scavenger-free ADCC. Values shown are % of control value (mean ± s.e.) with the number of experiments given in parentheses. ND, not determined.

of OH⁻ scavengers is our finding of intact antibody-dependent cell-mediated cytotoxicity (ADCC) activity in the presence of DMSO (150 mM), thiourea (50 mM), ethylene glycol (320 mM) or methanol (630 mM) (Table 1, protocol 3). The alterations in NK cell activity found with preincubation of LGL with OH⁻ scavengers (protocol 2) and the effect of OH⁻ scavengers on ADCC (protocol 3) were not significant by Student's *t*-test for two means. Another argument against a nonspecific toxicity of OH⁻ scavengers is our earlier finding of intact phytohaemagglutinin (PHA) and concanavalin A (Con A)-induced mitogenesis of human peripheral blood mononuclear cells in

the presence of OH⁻ scavengers at concentrations that resulted in the inhibition of NK cell activity¹¹.

A potential source of OH⁻ is the univalent reduction of molecular oxygen. In the monovalent pathway of oxygen reduction, superoxide anion (O₂⁻) is produced first, followed by the formation of hydrogen peroxide¹². O₂⁻ can interact with H₂O₂ to form OH⁻, as originally proposed by Haber and Weiss¹³. As O₂⁻ and H₂O₂ can serve as precursors of OH⁻, we examined the effect of SOD, a scavenger of O₂⁻, and catalase, a scavenger of H₂O₂, on NK cell activity. The results obtained by adding catalase (1,000 and 5,000 units ml⁻¹) or SOD (50 and 250 µg ml⁻¹), or catalase (5,000 units ml⁻¹) plus SOD (250 µg ml⁻¹) to the NK test system are summarized in Fig. 2. Catalase and SOD did not inhibit NK activity either alone or in combination. The inability of catalase to inhibit NK activity agrees with previous reports¹⁴. The lack of inhibitory effect of SOD on NK activity, however, does not support the findings of Roder *et al.*¹⁴, that SOD inhibited NK activity mediated by LGL using K-562 as target cells. In multiple experiments, we found that SOD, over a wide range of concentrations (50–250 µg ml⁻¹), failed to inhibit NK cell activity mediated by peripheral blood mononuclear cells, the non-adherent fraction of peripheral blood mononuclear cells or LGL, using either K-562 or MOLT-4 as target cells, in our experimental conditions.

The finding that OH⁻ scavengers inhibit NK cell activity, while scavengers of O₂⁻ and H₂O₂ (potential precursors of OH⁻) do not, suggests several possibilities. First, OH⁻ might be generated at the interplasma membrane space between the effector and target cells and catalase or SOD might not gain access to this space. Second, OH⁻ might be generated at an intracellular site;

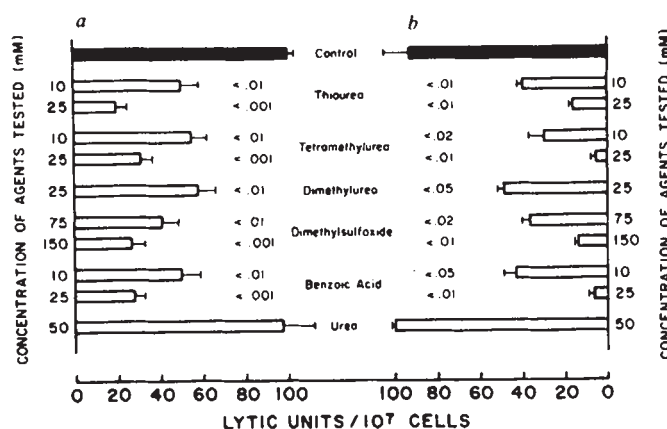


Fig. 1 Inhibition of NK cell activity by OH⁻ scavengers. Large granular lymphocytes (LGL) isolated from the non-adherent fractions of peripheral blood mononuclear cells by centrifugation on a discontinuous Percoll density gradient by the method of Timonen and Saksela²⁴ were used as effector cells. Adherent cell contaminating peripheral blood mononuclear cells were removed by passage over a Sephadex G-10 column as described previously²⁵. The NK cell preparations contained 0.1% monocytes or polymorphonuclear leukocytes as determined by nonspecific esterase staining²⁶ or by scoring LGL in a Neubaur Counting Chamber covered with an acridine orange-coated coverslip with simultaneous UV light and phase contrast illumination¹⁰. Cell lines, MOLT-4 or K-562, maintained as suspension cultures, were labelled with ⁵¹Cr and used as target cells in a 3-h ⁵¹Cr release assay²³. The per cent specific release (SCR) was calculated by the formula:

$$\frac{\text{experimental counts} - \text{spontaneous counts}}{\text{maximum counts}} \times 100$$

The cytotoxicity assay was established at multiple effector:target ratios and dose-response curves were generated by plotting per cent SCR versus the number of effector cells to compute lytic units. The number of effector cells needed to lyse 30% of the target cells was designated as one lytic unit. The effect of OH⁻ scavengers on NK cell activity was determined by adding various agents, at concentrations shown, to the cytotoxicity assay. Results are mean (±s.e.) of four experiments with MOLT-4 as target cells (a) and results of three experiments with K-562 as target cells (b). Statistical significance of inhibition mediated by OH⁻ scavengers was determined using Student's *t*-test for two means.

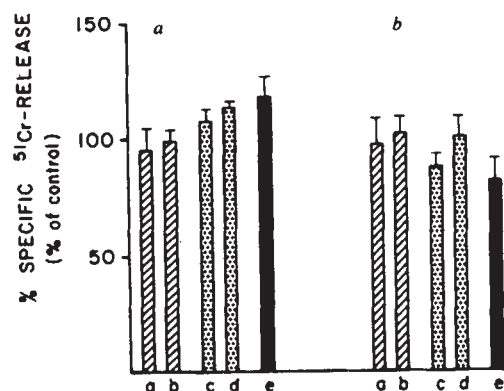


Fig. 2 Effect of catalase or SOD on human NK cell activity. a, Results of three experiments (mean ± s.e.) with LGL as effector cells and MOLT-4 as target cells. b, Results of five experiments with K-562 target cells. Effector:target ratio 25:1. a and b: catalase 1,000 and 5,000 units ml⁻¹ respectively; c and d: SOD 50 and 250 µg/ml respectively; e: catalase 5,000 units ml⁻¹ + SOD 250 µg ml⁻¹. Per cent of SCR without catalase or SOD (100% values) was 43 ± 2% with MOLT-4 as target cells and 38 ± 4% with K-562 as target cells.

Table 2 Effect of inhibitors of arachidonic acid metabolism on NK cell activity

Agent tested	Concentration (μM)	Target cells			
		K-562		Molt-4	
		Lytic units per 10^7 cells		Lytic units per 10^7 cells	
			P		P
None		174 $\pm$ 18		96 $\pm$ 6	
NDGA	10	46 $\pm$ 2	<0.01	23 $\pm$ 3	<0.01
NDGA	25	15 $\pm$ 4	<0.01	9 $\pm$ 2	<0.01
ETYA	10	130 $\pm$ 15	NS	65 $\pm$ 13	<0.02
ETYA	25	72 $\pm$ 13	<0.02	33 $\pm$ 3	<0.02
ETYA	50	38 $\pm$ 6	<0.01	ND	
Indomethacin	1	143 $\pm$ 16	NS	81 $\pm$ 10	NS
Indomethacin	10	138 $\pm$ 19	NS	71 $\pm$ 7	NS
Acetylsalicylic acid	100	149 $\pm$ 16	NS	85 $\pm$ 12	NS
Acetylsalicylic acid	1,000	135 $\pm$ 17	NS	73 $\pm$ 9	NS

LGL functioned as effector cells and ^{51}Cr -labelled K-562 or MOLT-4 were used as target cells. Inhibitors of arachidonic acid metabolism were added, at concentrations shown, to the NK test system. Lytic units were calculated as described in Fig. 1 legend. Results (mean $\pm$ s.e.) of three experiments. *P* determined by Student's *t*-test for two means. NS, not significant; ND, not determined.

a site that is also inaccessible to catalase or SOD. Third, $\text{OH}^\cdot$ might also be generated during arachidonic acid metabolism¹⁵. Besides being the source of $\text{OH}^\cdot$, additional interactions between $\text{OH}^\cdot$ and arachidonic acid can also be envisioned. Specifically, arachidonate may be oxidized by $\text{OH}^\cdot$ and the resulting metabolic products might be responsible for the cytolytic activity of NK cells. The findings that phospholipase A_2 is activated during human NK cell cytotoxicity¹⁶ and that horse eosinophil degranulation might depend on calcium-induced activation of phospholipase A_2 and generation of lipoxygenase products of arachidonic acid metabolism¹⁷, lend credence to the latter hypothesis. As arachidonic acid metabolism might be involved in NK cell activity either as a source of oxygen-centred radicals or of their metabolic products, either directly or indirectly, the effect of various inhibitors of arachidonic acid metabolism on NK cell activity was investigated. Nondihydroguaiaretic acid (NDGA), an inhibitor of the lipoxygenase pathway of arachidonic acid metabolism, and ETYA which inhibits both cyclooxygenase and lipoxygenase pathways, mediated significant inhibition of NK cell activity (Table 2). In contrast, acetylsalicylic acid and indomethacin, which inhibit the cyclooxygenase pathway, failed to mediate significant inhibition of NK activity.

How might $\text{OH}^\cdot$ participate in NK cell activity of LGL? The possibility that $\text{OH}^\cdot$ contributes directly to target cell lysis by being generated at the interplasma membrane space between target cells and effector cells cannot be rigorously excluded. However, involvement of $\text{OH}^\cdot$ in earlier steps in the sequence of events leading to target cell lysis seems more likely. This latter notion is supported by the findings of defective NK cell activity in Chediak-Higashi patients, whose cells generate reactive oxygen intermediates in response to NK-susceptible targets¹⁴ but are inefficient as NK cells, suggesting that free radical generation alone might not be sufficient to mediate cytotoxicity. In the context of a 'stimulus-secretion' model for NK cell activity^{9,18,19}, and in view of evidence that lysosomal enzymes may be the actual lytic moiety^{1,9,18}, $\text{OH}^\cdot$ participation may be via release of potent lysosomal enzymes either by direct lysosomal membrane peroxidation²⁰ or by a cyclic nucleotide-regulated mechanism^{21,22}. Alternatively, $\text{OH}^\cdot$ might also participate in NK cell cytotoxicity via lipoxygenase pathway products. Clearly, further work is required to elucidate the role of $\text{OH}^\cdot$ in the complex biochemical mechanisms leading to target cell lysis in the NK cell system. It is already clear, however, that $\text{OH}^\cdot$ or a closely related species of free radical is involved in the metabolic programme pertinent to acquisition of lytic potential by NK cells.

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Viral perturbation of endocrine function: disordered cell function leads to disturbed homeostasis and disease

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Virus-induced disease occurs both through direct destruction of cells by viruses and/or secondarily through lysis of infected cells by immunological assault¹⁻⁵. We asked whether viruses could also cause injury and disease by altering the cell's normal or expected functions without destroying the cells. Here we show that a relatively noncytopathic virus can perturb endocrine functions by disordering the synthesis of a hormone needed for growth and glucose regulation, while replicating in a specialized cell making that hormone. Yet, despite viral replication and alteration in synthesis of the specialized cell's homeostatic product, the infected cell remains free from structural injury.

Newborn C3H/St, C3H/HeJ, C57Br/cdj, CBA/N, AKR/J and BALB/kae nude mice infected with 60 plaque-forming units (PFU) of lymphocytic choriomeningitis virus (LCMV) Armstrong strain 1371 had clear-cut developmental retardation manifested as decreases in body weight and length⁶ that became evident as early as 5 to 7 days after viral inoculation. Pituitaries from infected mice had viral antigens but were structurally normal (Fig. 1A). Despite the presence of viruses in these cells, there was no evidence of altered anatomy, cell lysis or inflammatory infiltrates by low power microscopy; high resolution microscopy showed normal cell morphology and structure^{7,8}.

Using monoclonal antibodies⁹ directed against LCMV polypeptides, we demonstrated the restriction of viral replication to the pituitary glands' anterior lobe (Fig. 1B). Application of a two-fluorochrome system¹⁰ designed to stain LCMV nucleoprotein with one dye and either growth hormone (GH) or prolactin (PL) with the other revealed LCMV replicating primarily in those cells that made GH and not in cells making PL (Fig. 1C-G). Other studies showed that LCMV did not replicate in cells making thyroid stimulating hormone or adrenocorticotrophic hormone^{7,8}.

The two major activities of GH are regulation of growth and glucose metabolism^{11,12}. Accordingly, mice persistently infected with LCMV ARM 1371 underwent significant decreases in body length and weight and became defective in glucose metabolism (Table 1, Fig. 2). Simultaneously, GH levels diminished in pituitary glands of infected mice, but PL levels remained normal. Serum contains insulin-like growth factors (IGFs) at concentrations dependent on GH¹³. Measurement of IGF-I¹⁴ in serum from 16 individual LCMV-infected C3H/St mice yielded a concentration of $0.15 \pm 0.02 \text{ U ml}^{-1}$ (mean ± 1 s.e.) as compared with mean values from 20 uninfected matched controls of $0.45 \pm 0.04 \text{ U ml}^{-1}$ (d.f. 34, $P < 0.001$). These results further support a generalized GH deficiency. In the sera, cortisol and insulin levels were normal, but blood glucose decreased markedly (Table 1). The rise in blood sugar after glucose administration in LCMV-infected mice was greater than in uninfected mice (Fig. 2). Histopathological analysis of the pancreas, specifically islets of Langerhans cells, showed normal cell structure and morphology (Fig. 1H) by both light and electron microscopy in over 98% of the 500 islets studied. Approximately 2% of islets had cellular infiltrates, but with minimal necrosis. Viral antigen appeared both in acinar cells of the pancreatic ductal tissue and in the β cells of the islets of Langerhans. The amount of insulin in pancreases of LCMV-infected mice ($n = 5$, mean ± 1 s.e.: $0.33 \mu\text{U}$ of insulin per g of pancreas ± 0.02) was equivalent to that found in uninfected matched 15-day-old C3H/St controls (0.34 ± 0.04).

To demonstrate definitively the role of GH deficiency in the metabolic disorder of mice infected with LCMV, cloned GH-producing cells (GH-3)¹⁵ were transplanted into 6-day-old nude mice infected at birth with 100 PFU of LCMV. Figure 3 shows that the LCMV-infected recipients of GH grew normally and survived at the rate expected for uninfected age- and sex-matched controls. In contrast, infected littermates without GH transplants died from severe hypoglycaemia associated with their GH deficiency. Mice persistently infected with LCMV and with replaced GH had normal blood glucose levels (infected mice with replaced GH: blood sugar (mg per dl): mean ± 1 s.e.), 128 ± 5 , uninfected matched controls 134 ± 8 , $P = 0.4$, d.f. = 14), and normal glucose tolerance tests (Fig. 4). Hence, the severe disease was caused by GH deficiency associated with LCMV replication in GH-producing cells and was corrected by replacing GH in the virus-infected mice.

There are three primary ways in which viruses could alter cellular function. First, as a result of infection, the cell might release a product like interferon (IFN) that would modulate its function. However, IFN does not have a role in the GH deficiency due to LCMV infection. In our experiments, antibody to mouse IFN that significantly blocked IFN-associated glomerular membrane and liver disease associated with LCMV infection¹⁶⁻¹⁹ (F. J. Dutko and F. Chisari, manuscript in prepar-

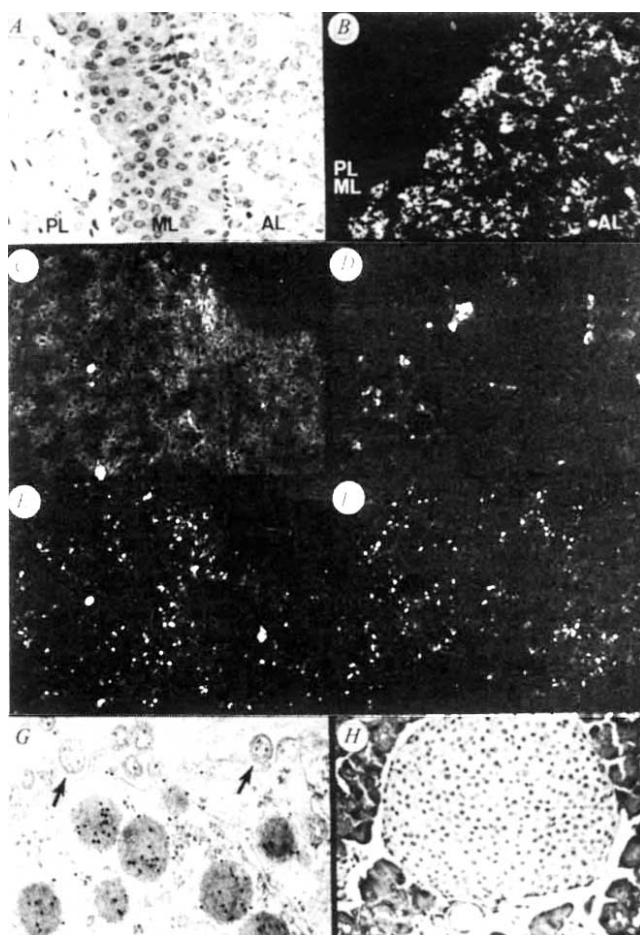


Fig. 1 Histopathological and immunochemical studies of endocrine tissues and cells taken from 15–18-day-old C3H/St mice persistently infected by injection at birth with 60 PFU of LCMV intracerebrally. Intraperitoneal inoculations gave corresponding findings. All mice carried infectious virus as determined by either PFU assay or intracerebral transfer of their sera into 4-week-old susceptible mice. **A**, Normal cell structure and relationships of anterior (AL), middle (ML) and posterior (PL) pituitary lobes. No cell destruction or inflammatory infiltrates are present. **B**, LCMV antigen expression is restricted to cells of the anterior lobe (AL) of the pituitary gland and not to the middle or posterior lobes. Four-micrometre cryostat-cut sections of the pituitary gland were stained with monoclonal antibody to LCMV nucleoprotein and fluorescein isothiocyanate (FITC) dye. Results were similar in over 50 pituitaries from individual LCMV-infected mice examined with these reagents or with a guinea pig polyclonal antibody to LCMV. **C**, **E** represent an identical tissue section. In **C**, tissue is stained with a monospecific antibody to growth hormone (GH) and FITC, while in **E**, tissue is stained with a monoclonal antibody to LCMV nucleoprotein and a rhodamine dye. Results were similar when a monoclonal antibody to mouse GH-FITC was substituted for the monkey antibody to mouse GH. Note similarity of staining pattern indicating that LCMV is replicating in cells making GH. **D**, **F** represent a tissue section stained either with monospecific antibody to prolactin (PL) and FITC or a monoclonal antibody to LCMV nucleoprotein and rhodamine dye (**F**). Note dissimilarity of staining pattern indicating that LCMV is not replicating in PL-containing cells. **G** Demonstrates the replication of LCMV in GH-containing cells of the anterior lobe of the pituitary gland. GH in secretory granules was labelled using monkey antibody to mouse GH and goat antibody to monkey IgG conjugated to gold particles ($\times 17,500$). Of over 100 cells studied in the pituitary gland, LCMV (arrows) budded from only those cells with GH-containing vesicles. **H** Illustrates the normal cytomorphology of islet of Langerhans in the pancreas and absence of cell lysis or inflammatory infiltrates. (For methods for plaquing virus, conjugation using two fluorochrome dyes, immunofluorescence, immunoelectron microscopy and light microscopy see refs 7, 8, 10, 25.)

Table 1 Perturbation of growth and glucose metabolism associated with persistent LCMV infection of cells producing growth hormone in the anterior pituitary

	No. of mice†	C3H/St mice* at					
		Day 5			Day 15		
		Uninf.	Inf.	P‡	Uninf.	Inf.	P
Growth							
Length (cm)	20	2.8±0.03‡	2.5±0.02	<0.01	4.2±0.05	3.3±0.08	<0.001
Weight (g)	50	3.3±0.1	3.2±0.1	NS	7.7±0.1	4.1±0.1	<0.001
Pituitary gland							
Growth hormone	12	5.3±1	6.0±1	NS	16.2±2	7.0±1	<0.001
Prolactin	6	0.3±0.02	0.3±0.03	NS	0.3±0.04	0.3±0.05	NS
Blood							
Glucose (ng dl ⁻¹)	34	ND	ND	ND	144±20	43±5	<0.001
Insulin (μU ml ⁻¹)	20	ND	ND	ND	28±2	35±5	NS
Insulin-like growth factor (U ml ⁻¹)	16	ND	ND	ND	0.45±0.04	0.15±0.02	<0.001
Cortisol (ng ml ⁻¹)	20	ND	ND	ND	11±1	9±1	NS
Glucose tolerance test (abnormal)	10	ND	ND	ND	0/12	10/10	<0.001
Sodium (mequiv. l ⁻¹)	15	ND	ND	ND	141±6	142±6	NS
Potassium (mequiv. l ⁻¹)	15	ND	ND	ND	5.6±0.1	5.1±0.2	NS

Results from the pituitary gland are in μg pituitary hormone per mg of pituitary gland. Pituitary hormones were isolated, using 10% acrylamide base gel and quantitated by radioimmune assay as described by Sinha^{22,23}. Mean values for individual pituitary glands were found. Blood samples from individual mice were assayed. Glucose, sodium and potassium concentrations were determined in 5 to 20 μl of serum using microautomatic Beckman Astra-8. Cortisol and insulin determinations were by radioimmune assay and a competitive inhibition technique. For glucose tolerance tests each mouse received 2 mg glucose per g of body weight, intraperitoneally²⁴. Samples obtained from each mouse before glucose challenge and 1 h afterward were tested for glucose and, when sufficient material was left, for insulin and cortisol levels. The numerator represents the number of mice showing a twofold or greater increment in glucose levels 1 h after a measured glucose challenge. The denominator shows the total number of mice studied. Radioimmune assay was used for insulin-like growth factor²⁰. NS, not significant; ND, not done.

* Within 18 h of birth, mice were inoculated with 60 PFU of LCMV Armstrong 1371.

† Minimal number of mice per group. ‡ Mean ± 1 s.e.m. § P value, NS, not significant difference.

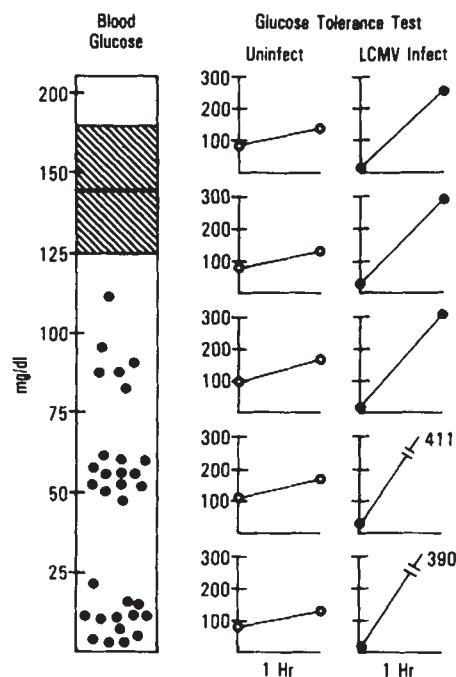


Fig. 2 Perturbation of glucose metabolism in 15-day-old C3H/St mice persistently infected with LCMV. On the left is the mean glucose value of 144 mg dl⁻¹ (heavy horizontal line) and 2 s.e. above and below mean (stripped areas) in 48 age- and sex-matched uninfected controls. The blood glucose values for individual C3H/St mice persistently infected with LCMV (●) are shown. On the right are 1-h glucose tolerance tests (GTT) performed on uninfected and LCMV infected mice. Of the 12 uninfected mice studied, none developed twofold increases by 1 or 2 h after receiving 2 mg glucose per g body weight. In contrast, all 10 LCMV-infected mice showed far greater than twofold enhancement in similar conditions. Values for five other LCMV-infected mice not diagrammed were: pre-glucose challenge–post-glucose challenge: 48–570; 60–356; 52–184; 56–334; 52–314.

ation) failed to alleviate deficiencies in either growth or glucose metabolism. Further, IFN levels in the pituitary gland were similar in virus-infected and uninfected matched controls (less than 1.2 units of IFN per g pituitary tissue). Second, the virus might alter cell function by changing the structure of GH. Peptide maps of GH developed by using trypsin or chymotrypsin digests failed to show any structural differences in GH isolated from pituitary glands of infected or uninfected mice (data not shown). Thus, LCMV does not appear to modify the GH polypeptide structurally, although there are no data as yet on the functional activity of these molecules. Third, and most likely, viruses may affect the synthesis of GH at transcriptional or translational levels. Thus, the cells' capacity for membrane translocation and synthesis may be limited so that production of viral gene products competes with manufacture of GH, thereby overloading the system. Alternatively, the virus may induce a specific alteration in an enzyme or transport system needed for the production and release of GH. Work is continuing to investigate these and other possibilities.

Considering the enormous variety of human diseases of which aetiologies are still unknown, it is interesting to speculate that many of these could be caused by failure of the specialized or differentiated function of a cell due to a virus infection. This would include disorders involving faulty manufacture of immune regulators, insulin or other hormones, or neurotransmitters. Others reported^{20,21} that viruses can lyse differentiated cells, thereby causing disease. In contrast, we show here that virus causes pathology without cytotoxic injury. This may signal a search for viruses in a variety of disorders even in the absence of defined anatomic or morphological changes.

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Fig. 3 Transplantation with GH-producing cells corrected the growth deficiency of mice persistently infected with LCMV and allowed a survival rate comparable with that of uninfected age- and sex-matched controls. For these experiments, littermates receiving LCMV at birth were segregated, at random, into three groups. One group received 1×10^7 GH-3 cells (making about 7,800 ng of GH per 24 h in culture) intraperitoneally, initially and twice weekly thereafter. A second group received intraperitoneally, either phosphate-buffered saline or cells not making GH. The third group was not inoculated at all. Each group contained a minimum of eight mice and was handled identically. ■, Uninfected control; □, LCMV-infected but not GH replaced; ▨, LCMV-infected and GH replaced. Cumulative survival of such 50-day-old uninfected mice and LCMV-infected mice reconstituted with GH exceeded 80%. In contrast, the survival rate of LCMV-infected mice not without replaced GH was <5%.

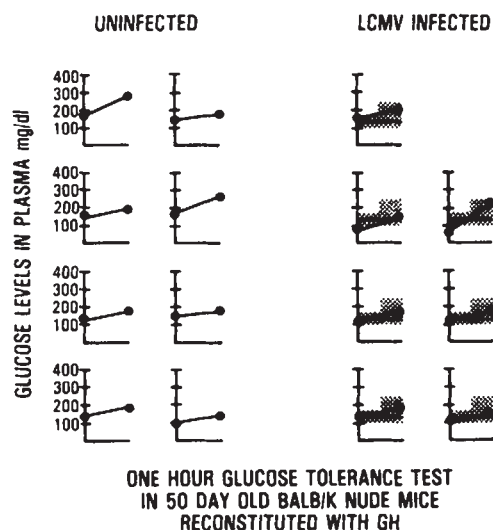
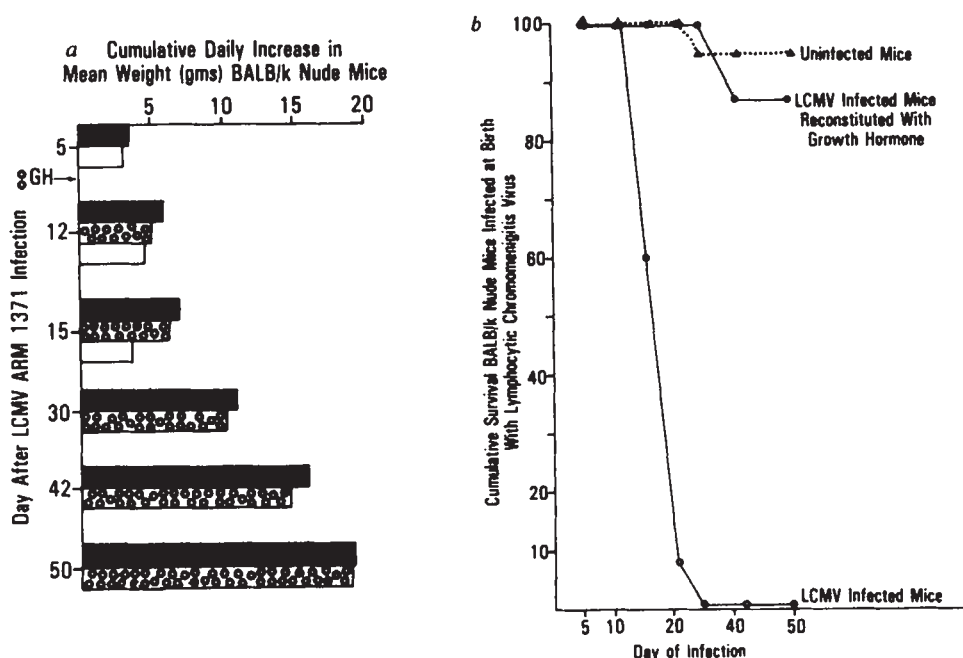


Fig. 4 Replacement with GH normalized the glucose metabolism of mice persistently infected with LCMV. Results of the glucose tolerance test consisted of pre-test samples compared with serum samples taken 1 h after challenge with 2 mg of glucose per g of body weight, intraperitoneally. LCMV-infected mice: horizontal bar represents the mean blood glucose levels of 10 uninfected age matched controls (2 not shown); the shaded area represents 2 s.e.m.

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Homologous cell-derived oncogenes in avian carcinoma virus MH2 and murine sarcoma virus 3611

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Retroviral oncogenes (*v-onc*) are derived from cellular genes (*c-onc*) which are highly conserved among different species¹⁻³. Retrovirus-transduced oncogenes are most commonly associated with the induction of haematopoietic tumours and sarcomas⁴. The avian retrovirus Mill Hill no. 2 (MH2) was isolated from a spontaneous ovarian tumour of a chicken and is distinguished by the predominant induction of liver and kidney carcinomas in fowl⁴⁻⁶. MH2 also induces transformation of fibroblasts, macrophages and epithelial cells in culture^{2,6,7}. The genome of MH2 contains two unrelated and independently expressed cell-derived oncogenes, *v-mil* and *v-myc*^{8,9}. Three other viral isolates among avian acute transforming retroviruses

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contain the *v-myc* oncogene^{2,10}, but only MH2 contains both *v-myc* and *v-mil*. Hence, some of the pathogenic specificities of MH2 may be due to the simultaneous expression of two oncogenes. The murine sarcoma virus 3611 (3611-MSV) isolated from a mouse carrying lung carcinoma and peritoneal tumours¹¹, induces fibrosarcomas in newborn mice and the transformation of fibroblasts and epithelial cells in culture^{11,12}. The oncogenic properties of 3611-MSV are due to the presence in its genome of a cell-derived oncogene termed *v-raf*¹³. We report here that the two independently transduced oncogenes *v-mil* and *v-raf* are closely related and that they were apparently derived from homologous cellular genes of avian and mammalian species.

The first indication of a possible sequence relationship between cloned *v-mil* and *v-raf* DNAs came from a comparison of their restriction site patterns (Fig. 1). Both genes have a complexity of ~1.2 kilobases (kb) and share *Sph*I and *Stu*I sites in their central regions. Relative to these sites, the 5' and 3' borders of these genes seem to be non-overlapping. Figure 2 provides direct demonstration of sequence homology. Cloned MH2 DNA was digested with appropriate restriction enzymes, and blotted DNA fragments were hybridized to both *v-mil* and *v-raf* probes (Fig. 2a-c). All *mil*-containing fragments strongly hybridized with the *v-mil*-specific probe, including fragments resulting from partial digestion and undigested 8.0-kb DNA (Fig. 2b). Using the *v-raf*-specific probe, hybridization was observed with basically the same fragments (Fig. 2c). The differential intensity of hybridization of specific fragments in the relatively stringent conditions used allowed us to make some conclusions about the regions of strong homology; especially informative was the hybridization of partially and completely digested fragments obtained from digestion with *Stu*I. The 4.60-kb and 3.84-kb fragments of integrated MH2 DNA resulting from incomplete digestion with *Stu*I revealed stronger hybridization to the *v-mil* probe than the fragments of 4.16 and 3.40 kb resulting from complete digestion (Fig. 2b), because the larger fragments contain more sequences homologous to the *v-mil* probe. This effect was much more pronounced, however, when the *v-raf* probe was used (Fig. 2c), and the 5' 4.16-kb fragment did not hybridize at all in the conditions used. This implies that the central region of *v-mil*, around the *Sph*I site, contains sequences closely homologous to *v-raf*, while the 5' and 3' regions are less homologous, in good agreement with the restriction patterns shown in Fig. 1. Complete confirmation of these conclusions was obtained by digestion of cloned 3611-MSV DNA with appropriate restriction enzymes and hybridization of blotted DNA fragments with both the *v-raf* and *v-mil* probes (Fig. 2d-f). Again, fragments containing the central region of *v-raf* around the *Sph*I site, like the 0.50-kb *Hpa*I/*Sst*II fragment, showed the strongest hybridization with the *v-mil* probe (Fig. 2f).

Figure 3 shows a heteroduplex analysis between cloned integrated MH2 DNA and a *v-raf*-containing segment of 3611-MSV DNA subcloned in pBR322. Reannealing of denatured DNAs was done in conditions of low stringency and for short periods of time to allow isolation of heteroduplex structures formed between sequences of moderate homology; *v-raf* and *v-mil* sequences annealed over a contiguous segment of ~0.9 kb. The *v-raf*-containing DNA fragment contained about 0.27 kb of pBR322 sequences 5' from the *Xho*I site of *v-raf* (compare with Fig. 1); since the 5' single-stranded region (d) of the *v-raf*-containing DNA in the heteroduplex structure was approximately of that length, it seems that homology to *v-mil* starts near the *Xho*I site of *v-raf* and continues for ~0.9 kb. This is in agreement with the alignment of the two genes relative to the conserved *Sph*I site (Fig. 1) and with the pattern of hybridization obtained between cloned *v-mil* and *v-raf* DNAs in Southern blots (Fig. 2).

Chicken and human genetic loci related to *v-mil* and *v-raf*, respectively, have been molecularly cloned and characterized (refs 9, 14 and H.W.J., K.B., T.I.B. and U.R.R., unpublished data). The relationship of *v-mil* and *v-raf* to the chicken and

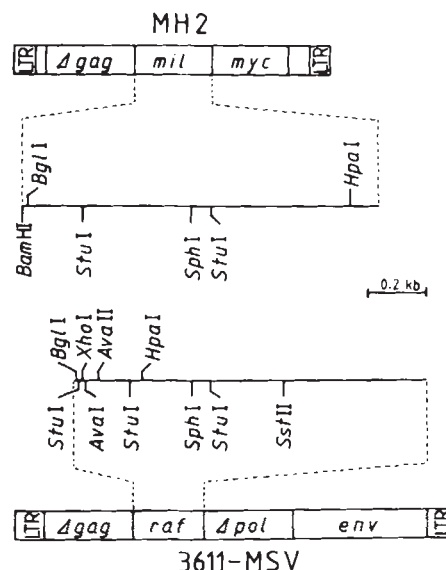


Fig. 1 Comparison of the restriction site patterns of the avian oncogene *v-mil* and the murine oncogene *v-raf*. The location of these genes on the genetic maps of cloned proviral DNAs of MH2^{8,9} and 3611-MSV¹³, is shown at the top and bottom, respectively. Both genes are resistant to the following restriction endonucleases: *Bgl*II, *Cl*aI, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Mlu*I, *Pvu*I, *Pvu*II, *Sst*I, *Sal*I, *Sma*I and *Xba*I.

human genes is shown in Fig. 4. DNA of the λ phage clone *c-mil*-4, containing the single-copy chicken locus related to *v-mil*⁹, and of a pBR322 clone containing the single active human homologue of *v-raf*, hu *c-raf*-1¹⁴, were digested with appropriate restriction enzymes. Blotted DNA fragments were hybridized to the *v-mil* and the *v-raf* probe in conditions of high and low stringency (Fig. 4b-e). From an *Eco*RI/*Bam*HI digest of *c-mil*-4 DNA, three fragments of 10.0, 2.2 and 1.0 kb hybridized with the *v-mil*-specific probe to a similar extent at high and low stringency, confirming the close homology⁹. In contrast, the *v-raf* probe hybridized only to the 10.0-kb fragment at high and low stringency conditions, and in addition very weakly to the 1.0 kb fragment in low stringency conditions. The 2.2-kb fragment, containing the *c-mil* exon homologous to the extreme 5' sequences of *v-mil*, did not hybridize at all with *v-raf*, in agreement with the data shown in Figs 1-3. From an *Eco*RI/*Sal*I digest of hu *c-raf*-1 cloned in pBR322, three fragments of 4.8, 3.0 and 0.7 kb hybridized with the *v-raf*-specific probe equally well in high and low stringency conditions, and an additional 2.8-kb fragment hybridized weakly in the relaxed conditions (the 3.7-kb *Eco*RI/*Sal*I fragment of pBR322 hybridized to the pBR322 sequences in the *v-raf* probe). With the *v-mil*-specific probe, the 4.8- and 3.0-kb fragments hybridized in stringent conditions and even stronger in relaxed conditions, and the 0.7-kb fragment hybridized only in low stringency conditions. In general, the data in Fig. 4 confirm the relationship between *v-mil* and *v-raf* demonstrated above, and strongly suggest that these two viral oncogenes were derived from homologous genetic loci. This is also supported by the observation that the same fragments of total digested genomic DNA either of chicken or of human origin hybridized to both the *v-mil* and the *v-raf* probe (not shown). Furthermore, the exon-intron arrangements of the human *c-raf* and the chicken *c-mil* loci seem very similar (refs 9, 14 and T.I.B., U.R.R., H.W.J. and K.B., unpublished data).

The extensive sequence homology reported here for the avian oncogene *v-mil* and the murine oncogene *v-raf* strongly supports the view that these two genes were derived from homologous genetic loci of avian and mammalian species. The totally independent transductions of these genes in two highly oncogenic retroviruses of different species directly indicates that the *mil/raf* gene family is associated with potential oncogenic

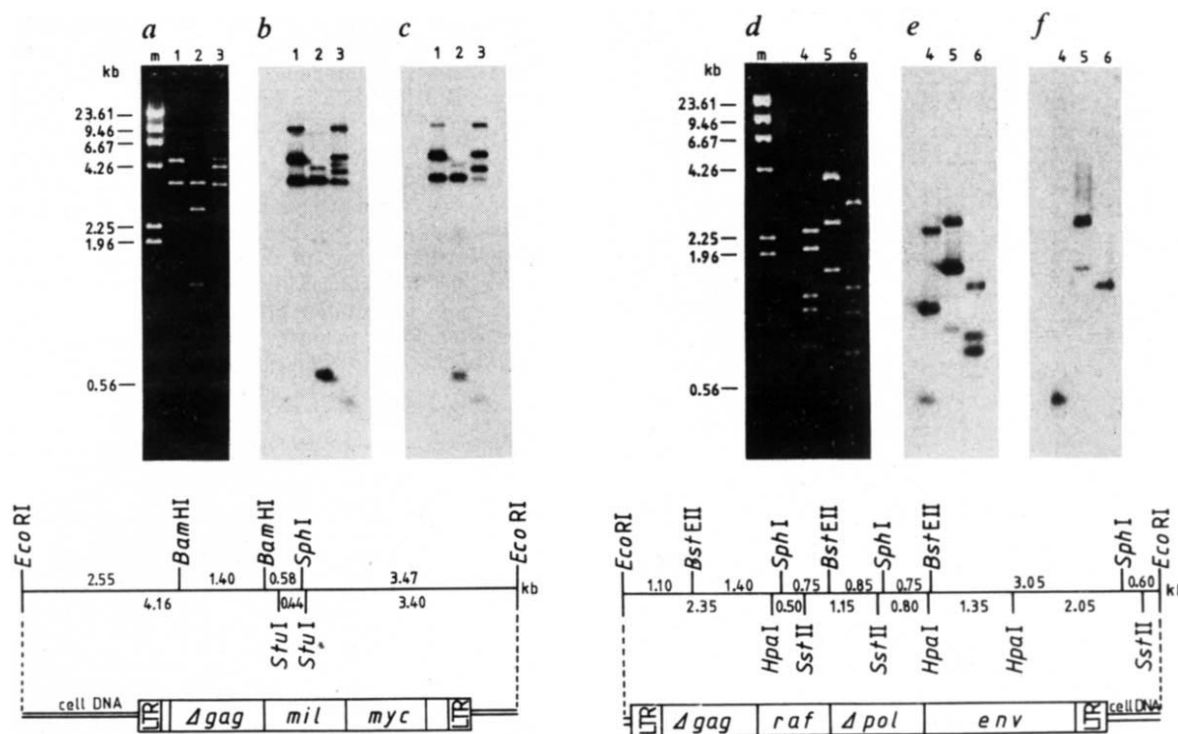


Fig. 2 Detection of sequence homology between *v-mil* and *v-raf*. **a**, The purified 8.0-kb *EcoRI* fragment of phage clone λ MH2-1 (refs 8, 9), containing the entire MH2 provirus flanked by quail cellular sequences, was digested with *SphI* (lane 1), *SphI* and *BamHI* (lane 2), or *StuI* (lane 3). The digests were separated in two parallel sets by electrophoresis through a 1.25% agarose gel using a *HindIII* digest of λ c1857S7 as molecular weight standard (m). Faint bands visible in the ethidium bromide stain result from incomplete digestion of the 8.0-kb fragment. The location and complexities (in kb) of the fragments obtained by these digestions are shown below in the physical map of MH2 DNA. DNA was transferred to nitrocellulose sheets and hybridized with either **b**, a *v-mil*-specific probe, represented by the gel-purified 1.1-kb *BamHI*/*HpaI* fragment of cloned MH2 DNA^{8,9} (compare with Fig. 1); or **c**, a *v-raf*-specific probe, represented by the gel-purified 1.8-kb *BglII* fragment of a pBR322 plasmid containing in its *SalI* and *HindIII* sites a 1.9-kb *XhoI*/*HindIII* fragment of cloned 3611-MSV DNA extending from the 5' end of *v-raf* into the Δpol sequences¹³ (compare with Fig. 1). The purified 1.8-kb *BglII* fragment used as a probe contains 0.27 kb of pBR322, all the *v-raf* sequences 3' of the *XhoI* site and 0.5 kb of Δpol sequences. Probes were radiolabelled by nick-translation using [α -³²P]dCTP to a specific activity of $\sim 5 \times 10^7$ c.p.m. μg^{-1} . Hybridization was at 42 °C for 18 h in a solution containing 50% (v/v) formamide, 5 $\times$ SSC (1 $\times$ SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 10 $\times$ Denhardt's solution (0.2% Ficoll, 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone), 0.1% SDS, 100 $\mu\text{g ml}^{-1}$ denatured DNA from herring testes, and 1.5×10^7 c.p.m. of the corresponding probes. Filters were washed three times for 45 min each at 65 °C in a solution containing 2 $\times$ SSC and 0.1% SDS. **d**, The purified 8.5-kb *EcoRI* fragment of a pBR322 plasmid containing the entire 3611-MSV provirus flanked by mouse cellular sequences¹³ was digested with *HpaI* and *SstII* (lane 4), *SphI* (lane 5), or *SphI* and *BstEII* (lane 6). The location and complexities of the fragments obtained by these digestions are shown below on the physical map of 3611-MSV DNA. Electrophoresis of the digests, transfer of DNA, and hybridization with: **e**, the *v-raf* probe; or **f**, the *v-mil* probe, was as described above.

function. A similar close relationship has been reported between the transforming genes of three different isolates of feline sarcoma viruses and the oncogenes of avian Fujinami sarcoma virus, simian sarcoma virus, or Abelson murine leukaemia virus, respectively¹⁵⁻¹⁷. Some of these genes belong to a family of oncogenes specifying tyrosine-specific protein kinases¹. In contrast, no enzymatic activity has been demonstrated yet for the products of the *v-mil* and *v-raf* genes, which are both expressed as *gag*-related hybrid proteins^{8,9,11,13}. The oncogenic properties of MH2 and 3611-MSV suggest that the *mil/raf* gene family may be involved in epithelial cell transformation. Both viruses transform such cells in culture, and MH2 induces a high

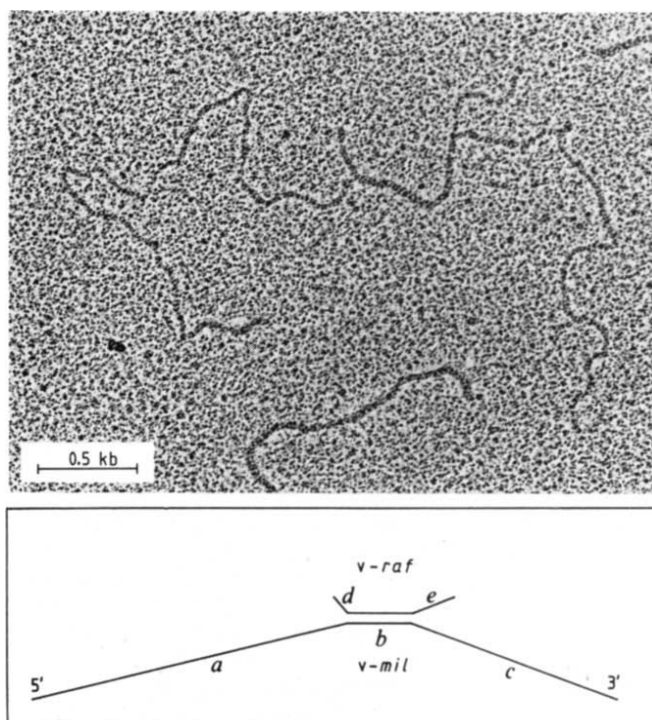


Fig. 3 Homology between *v-mil* and *v-raf* demonstrated by heteroduplex analysis. The purified 8.0-kb *EcoRI* fragment of λ MH2-1 DNA containing the entire MH2 provirus (compare with Fig. 2) and the purified 1.8-kb *BglII* fragment containing most of the *v-raf* sequences (see Fig. 2 legend) were used for the analysis, which was done essentially as described previously⁹ except that the following parameters were modified: after denaturation of the DNAs, 2 M sodium perchlorate was added to a final concentration of 400 mM and renaturation was for 3 min only. The contour lengths (in kb) of single- and double-stranded regions and their standard deviations were determined: **a**, 4.22 ± 0.31 ; **b**, 0.92 ± 0.08 ; **c**, 3.03 ± 0.32 ; **d**, 0.23 ± 0.04 ; **e**, 0.59 ± 0.08 . From 44 to 53 heteroduplex structures of this type were analysed for the individual length measurements.

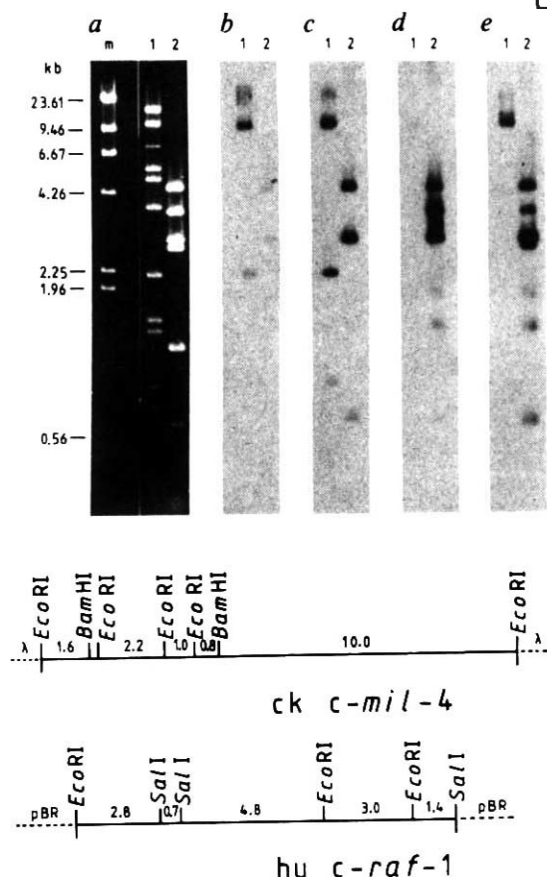


Fig. 4 Sequence relationship of the chicken locus *c-mil* and the human locus *c-raf* to both *v-mil* and *v-raf*. **a**, DNA of λ phage clone *ck c-mil-4*, carrying a 15.9-kb segment of the chicken genome including sequences homologous to all of *v-mil*⁹, was digested with *EcoRI* and *BamHI* (lane 1). DNA of a pBR322 plasmid carrying a 12.7-kb fragment of the human genome including sequences homologous to all of *v-raf* (*hu c-raf-1*)¹⁴ was digested with *EcoRI* and *SalI* (lane 2). The digests were separated in four parallel sets by electrophoresis through a 1% agarose gel using molecular weight standards (**m**) as described in Fig. 2 legend. The locations and complexities (in kb) of the fragments obtained are shown on the simple restriction maps of the two cloned loci. DNA was transferred to nitrocellulose sheets and hybridized with the *v-mil* (**b, c**) or the *v-raf* (**d, e**) probes described in Fig. 2 legend. Hybridization with 2×10^6 c.p.m. of the respective probes was for 4 h in conditions of higher (**b, d**) or lower (**c, e**) stringency. For higher stringency, conditions were like those described in Fig. 2 legend. For lower stringency, hybridization was at 60°C in a solution containing $3 \times \text{SSC}$, $10 \times \text{Denhardt's}$ solution, 0.1% SDS and $100 \mu\text{g ml}^{-1}$ denatured DNA from herring testes. Filters were washed at 60°C in $2 \times \text{SSC}$ (**b, d**) or $3 \times \text{SSC}$ (**c, e**) containing 0.1% SDS. The autoradiographs were exposed for 30 min using intensifying screens.

incidence of liver and kidney carcinomas *in vivo*. Also, the human homologue of the *mil/raf* gene family is located on chromosome 3 (ref. 14) which is specifically rearranged in small-cell lung carcinoma, certain cases of familial renal carcinoma, and mixed parotid gland tumours¹⁸⁻²². We can now analyse whether the human *mil/raf* locus is involved in such specific rearrangements.

We have shown previously that the chicken cellular homologues of the two oncogenes of MH2, *c-mil* and *c-myc*, do not map in close proximity to each other on the chicken genome⁹. We now know that the human *c-mil/raf* gene is located on chromosome 3 (ref. 14), whereas the human *c-myc* gene maps to chromosome 8 (ref. 22). Hence, two unrelated and unlinked cellular genes have been brought together in the MH2 genome. The mechanism of such a dual transduction is unknown, but it is possible that before transduction, the *mil* and *myc* loci had

been brought together by chromosomal translocations²² in the primary tumour from which the virus was isolated. Alternatively, two independent recombinational events between the transducing virus and the cellular genome could have occurred. The presence of two oncogenes on one retroviral genome opens the possibility that these genes may function cooperatively in the induction of specific tumours. Such a synergistic action between oncogenes has been suggested for the cell transformation by another avian retrovirus carrying two oncogenes, avian erythroblastosis virus²³, and for the transformation of primary cell cultures by transfection with pairs of oncogenes belonging to different complementation groups^{24,25}.

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Cell lineage-specific undermethylation of mouse repetitive DNA

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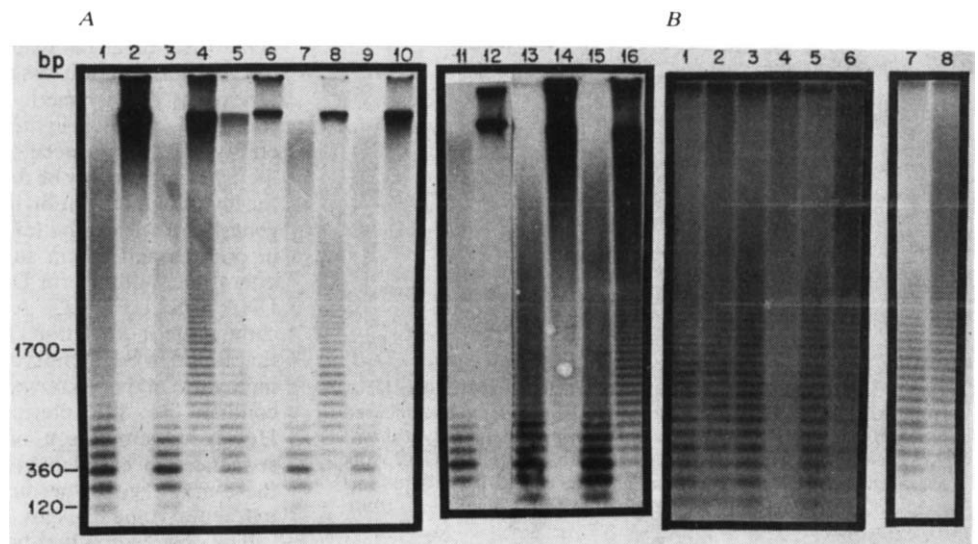
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Several distinct cell lineages are established during mouse embryogenesis. The trophoblast and primitive endoderm give rise to extraembryonic structures alone, while the primitive ectoderm becomes the fetus proper¹⁻³. Recent studies suggest that the levels of DNA modification are lower in inactive X chromosomes from extraembryonic tissues than in embryonic and adult somatic tissues. Using *HpaII/MspI* isoschizomers, Southern blots and cloned probes, we show here that repetitive DNA sequences from all derivatives of the two extraembryonic lineages, trophoblast and primitive endoderm, are substantially undermethylated compared with primitive ectoderm derivatives. This contrasts with the highly methylated state of these repetitive elements observed in adult somatic tissues. Specific demethylation or inhibition of *de novo* methylation, or a combination of both mechanisms, may be involved. These findings suggest that elements of gene regulation dependent on DNA modification may be different in extraembryonic cell lineages.

Fig. 1 Southern blot²⁵ probed with minor satellite sequence (pMR150) for DNA from various sources. Odd-numbered lanes, *Msp*I; even-numbered lanes, *Hpa*II digestion. A: lanes 1, 2, liver; 3, 4, sperm; 5, 6, day 7.5 embryonic tissue; 7, 8, day 7.5 ectoplacental cone (epc); 9, 10, day 7.5 decidua cells; 11, 12, day 7.5 embryonic tissue; 13, 14, day 15 yolk sac mesoderm; 15, 16, day 15 yolk sac endoderm. B: lanes 1, 2, day 7.5 extraembryonic ectoderm (ex); 3, 4, day 7.5 embryonic tissue; 5, 6, day 7.5 ectoplacental cone (epc); 7, 8, day 12 parietal endoderm. All tissues are from Ha/ICR randomly bred mice. Derivatives of the trophoblast were obtained from 7.5-day conceptuses, at which stage these tissues can still be isolated free of contaminating ICM-derived tissues. Conceptuses at 7.5 days post-coitum were dissected into embryonic, extraembryonic and



ectoplacental cone fractions in phosphate-buffered saline. The extraembryonic regions were further treated with pancreatin (2.5%; Sigma) and trypsin (0.5% in Ca^{2+} , Mg^{2+} -free saline) at 4 °C for 10 min to isolate the extraembryonic ectoderm²⁶. Decidual tissue from the day 7.5 conceptuses was also retained for DNA extraction. Derivatives of the primitive endoderm were separated from 12–15-day conceptuses. Parietal endoderm and Reichert's membrane were peeled away from the overlying giant cells⁴. Visceral endoderm was obtained by enzyme separation from the visceral yolk sac⁹. The remaining mesoderm of the yolk sac was also retained. Mature sperm were isolated from cauda epididymides. All tissues were centrifuged, and the saline was replaced by Pronase buffer (50 mM Tris-HCl, pH 10, 0.15 M NaCl, 0.1 M EDTA). Liver was Dounce-homogenized in 5% sucrose-TKM (50 mM Tris-HCl, pH 7.5, 24 mM KCl, 5 mM MgCl_2) buffer, and nuclei were isolated by centrifugation through a 20% sucrose-TKM cushion (5,000 r.p.m., 10 min). The pellet was resuspended in Pronase buffer. Tissue samples were incubated at 37 °C for 30 min in Pronase buffer with 1 mM dithioerythritol (DTE; Sigma). SDS (0.2% final concentration) and preboiled RNase A (150 $\mu\text{g ml}^{-1}$ final concentration; Boehringer Mannheim) were added and cells incubated at 37 °C for 1.5 h. The solution was made up to 0.8% SDS and 150 $\mu\text{g ml}^{-1}$ Pronase (Calbiochem) and incubated for 4 h at 37 °C. The digests were extracted twice with phenol (BRL ultrapure) and three times with chloroform/isoamyl alcohol (24:1). DNA was ethanol-precipitated, desiccated and resuspended in sterile water. DNA yield estimates are 6, 15 and 14 μg from ex, epc and embryo populations respectively for 30 7.5-day conceptuses and 200 μg parietal endoderm DNA from 68 12-day conceptuses. Yolk sac DNA yields are as previously reported¹³. Sperm DNA (800 μg) was extracted from free-swimming sperm released from four minced cauda epididymides. DNA was cut with restriction endonucleases *Msp*I (NE Biolabs) and *Hpa*II (BRL) in conditions recommended by the suppliers. Following overnight incubation, λ DNA was added and incubated for a further 6 h. Restricted DNA (0.5 μg per lane) was subjected to electrophoresis in 0.8% SeaKem Agarose gels using 36 mM Tris, 30 mM NaH_2PO_4 , 1 mM EDTA pH 7.4 buffer. To confirm complete digestion, we visualized limit-digest λ DNA fragments on the ethidium bromide-stained gel. The DNA was denatured, transferred to nitrocellulose (Schleicher and Schuell 85) and prehybridized in 4 × SSC, 5 × Denhardt's solution²⁷, 0.1% SDS, 0.1% Na pyrophosphate and 150 $\mu\text{g ml}^{-1}$ denatured herring sperm DNA for 4 h at 65 °C. ³²P (Amersham)-labelled nick-translated²⁸ pMR150 (specific activity 2×10^8 c.p.m. μg^{-1} , 20×10^6 c.p.m. per blot) was added and hybridized overnight. Filters were washed in 0.5 × SSC, 0.1% SDS at 65 °C and radiolabelled bands were visualized after 4 days by autoradiography (XAR-5 Kodak film).

The early stages of mouse embryogenesis are characterized by the establishment of several distinct cell lineages. By 4.5 days of development, two specialized lineages, trophoblast and primitive endoderm, have been set aside from the pluripotent primitive ectoderm⁴, and it is clear that cells are irrevocably committed to separate cell lineages by the late blastocyst stage^{5–7}. Studies of X-inactivation suggest that processes controlling gene expression in the specialized trophoblast and primitive endoderm lineages may differ from those found in pluripotent primitive ectoderm. Derivatives of trophoblast and primitive endoderm in the developing conceptus share the feature of preferential expression of the maternal X chromosome, whereas primitive ectoderm derivatives undergo random X-inactivation^{8–11}. Recent studies using DNA-mediated gene transfer have demonstrated that the DNA of the inactive X chromosome of adult somatic tissues seems to be modified and relatively inefficient in DNA transformation¹². By contrast, the inactive paternal X chromosome DNA of the visceral yolk sac endoderm (a primitive endoderm derivative) is relatively efficient in transformation and, therefore, is presumed to be less modified¹³. One possible explanation for these findings is that the general levels of DNA modification are lower in extraembryonic tissues than in embryonic and later adult somatic tissues. We have examined the levels of methylation at CpG sites at CCGG tetramers in the DNA of derivatives of different early cell lineages using probes for dispersed and centromeric repetitive DNA families. Comparison of the restriction patterns generated by the restriction endonuclease *Msp*I and the methyl-

cytosine-sensitive isoschizomer, *Hpa*II, reveals that repetitive DNA from all derivatives of the two extraembryonic lineages, trophoblast and primitive endoderm, was substantially undermethylated compared with primitive ectoderm derivatives and adult somatic tissues.

Several families of repetitive DNA have been identified from a library of cloned mouse (*Mus musculus*) middle repetitive DNA sequences¹⁴. Two of these cloned probes, pMR150 and pMR134 (pMR = plasmid mouse repetitive), identify families which are characterized by well defined patterns in Southern blots following restriction with *Msp*I. The sequences identified by pMR150 (minor satellite) (Fig. 1) are centromeric and are present in the genome as tandemly repeating elements of 120 base pairs (bp) with $\sim 5 \times 10^4$ copies. By contrast, pMR134 (*Mus* interspersed family, MIF) sequences (Fig. 2) are dispersed throughout the genome with $\sim 30,000$ copies of a 5–7-kilobase (kb) element^{15–18}. Both of these sequences seem to be fully methylated at CpG sites in adult liver (Fig. 1, lane 2; Fig. 2, lane 2).

The restriction patterns of *Msp*I and *Hpa*II digested DNA from ectoplacental cone (epc) and extraembryonic ectoderm (ex), which are post-implantation derivatives of trophoblast, are shown for minor satellite sequences in Fig. 1A, lanes 7, 8 and B, lanes 1, 2. These patterns indicate that a portion of the CpG sites of minor satellites are undermethylated relative to the amount observed in adult liver. By contrast, the DNA from the embryonic portion of the 7.5 day conceptus (Fig. 1, lanes 5, 6, 11, 12) does not seem to be digested by *Hpa*II and produces

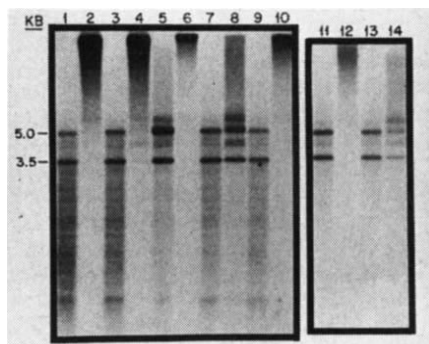


Fig. 2 Southern blot²⁵ probed with pMR134 (MIF) for DNA from various sources. Odd-numbered lanes, *Msp*I; even-numbered lanes, *Hpa*II digestion. 1, 2, liver; 3, 4, sperm; 5, 6, 7.5-day embryo; 7, 8, ectoplacental cone (epc); 9, 10, decidua cells; 11, 12, yolk sac mesoderm; 13, 14, yolk sac endoderm. All procedures and suppliers were as described in Fig. 1 legend.

a pattern comparable with that observed in adult liver (Fig. 1A, lane 2). An unusual *Msp*I pattern of the minor satellite sequences was observed in our original experiments with embryonic DNA (Fig. 1A, lane 5). Reanalysis of *Msp*I restriction of embryonic minor satellite sequences suggests that the pattern seen in the first experiment was due to incomplete digestion (Fig. 1A, lane 11). This finding suggests that our assessment of the relative *Hpa*II digestion of minor satellite sequences in liver and embryonic DNAs is valid. Maternal decidua cells, which surround the embryo in the uterus, also show extensive methylation of the minor satellite sequences (Fig. 1A, lanes 9, 10). *Hpa*II digestion of genomic DNA probed with MIF indicates that the undermethylation observed in epc and ex is not confined to the minor satellite sequences. The level of *Hpa*II digestion of DNA probed with MIF in epc is almost comparable with that produced by *Msp*I (Fig. 2, lanes 7, 8). Embryonic DNA from the 7.5 day conceptus shows no discernible restriction with the methyl-sensitive *Hpa*II (Fig. 2, lanes 5, 6).

We also examined the relative methylation of minor satellite and MIF CpG sites in parietal endoderm from day 12 conceptuses and visceral endoderm dissected from day 15 conceptuses. These two tissues are the later derivatives of the primitive endoderm^{4,19}. The Southern blot patterns for minor satellite sequences of *Hpa*II-restricted DNA from these tissues are similar to those observed for epc and ex (Fig. 1B, lanes 7, 8 and A, lanes 15, 16). The relative digestion of MIF sequences by *Hpa*II also shows significant undermethylation but the levels may be different from those observed in trophectoderm derivatives (Fig. 2, lanes 13, 14). By contrast, the DNA of the yolk sac mesoderm, which is a primitive ectoderm derivative, shows levels of methylation comparable with those seen in embryonic DNA (Fig. 1A, lanes 13, 14; Fig. 2, lanes 11, 12). Some *Hpa*II digestion of minor satellite sequences is detectable in the mesodermal extracts (Fig. 1A, lane 14). The degree of *Hpa*II digestion is less than that seen in visceral endoderm DNA and could be due to either contamination of mesodermal extracts with endoderm cells during enzyme separation of the two cell layers from yolk sac, or the fact that minor satellite sequences are partially undermethylated compared with embryonic DNA. Previous studies with X-chromosome expression in yolk sac derivatives⁹ suggest the first explanation but we cannot rule out the second possibility.

These studies indicate that both centromeric and dispersed repetitive DNA sequences of the mouse are undermethylated in the derivatives of cell lineages differentiated early in embryogenesis, that is, the trophectoderm and the primitive endoderm. By contrast, these same sequences appear to be fully methylated at 7.5 days of development in the primitive ectoderm cell lineage, which gives rise to the embryo. A previous study of methylation levels in total DNA of rabbit embryos also reported under-

methylation in trophectoderm²⁰. This was attributed to specific demethylation as the trophoblast DNA had the same degree of methylation as adult tissues whereas the embryonic ectoderm DNA was heavily methylated. Whether undermethylation of repetitive sequences in the extraembryonic lineages in the mouse embryo is due to specific demethylation or failure of *de novo* methylation can only be determined unequivocally by examining the levels of methylation in DNA from earlier stages of embryogenesis. However, the levels of methylation of these sequences in oocytes and sperm suggest that both mechanisms may be operating. Both sperm DNA (Fig. 1A, lanes 3, 4) and oocyte DNA (J.S., L.F., V.C., A. Chandley and N.H., in preparation) show undermethylation of minor satellite sequences similar to that found in extraembryonic tissues, suggesting that the *de novo* methylase activity known to occur in early embryos²¹ may be confined to the pluripotent primitive ectoderm lineage. However, comparison of the levels of methylation of MIF sequences in extraembryonic tissues and sperm suggests that there may also be specific demethylation of some CpG sites in extraembryonic lineages.

The observation that both minor satellite and MIF sequences are undermethylated in certain extraembryonic lineages argues that the undermethylation is not limited to either a particular class of repetitive sequences or a particular chromosomal localization. These data further suggest that the general levels of DNA methylation may be reduced for the entire genome and that elements of gene regulation dependent on DNA modification may differ in these extraembryonic cell lineages. Such differences could conceivably account for the differences in DNA transfer of inactive X-chromosome genes from yolk sac¹³ and the undermethylation of human globin genes in placental DNA²². It is also conceivable that the presence of some cellular oncogene transcripts in extraembryonic lineages²³ and the expression of endogenous retroviruses in placenta²⁴ are a function of the undermethylation of DNA in these tissues. Study of the levels of methylation of these and other structural genes in the extraembryonic lineages will determine whether undermethylation is a widespread phenomenon in these tissues. These studies will have considerable implications for the role of DNA modifications in cell lineage development and differentiation.

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Regulation of *Antennapedia* transcript distribution by the *bithorax* complex in *Drosophila*

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The homoeotic genes of *Drosophila* seem to be involved in the establishment of developmental pathways and the specification of different anatomical segment identities^{1,2}. For example, it has been proposed that a function of the *Antennapedia*⁺ (*Antp*⁺) locus is to direct embryonic cells to follow a mesothoracic developmental pathway³⁻⁵. Using cloned cDNA probes of the *Antp*⁺ (ref. 6) locus and an improved *in situ* hybridization method⁷, we found that the neural cells of the embryonic and larval mesothorax possess higher levels of *Antp*⁺ transcripts than the neural tissue of the other segments^{7,8}. Here we present further evidence that *Antp*⁺ products are associated with formation of the mesothorax, obtained by analysing the distribution of *Antp*⁺ transcripts in *bithorax* mutants. Embryos homozygous for any one of several *bithorax* mutations display a transformation of the epidermis of the more posterior segments into the homologous tissue of the mesothorax¹. For example, the metathorax and first seven abdominal segments of embryos that completely lack the *bithorax* gene complex show a cuticular phenotype characteristic of the normal mesothorax^{1,9}. By *in situ* hybridization of an *Antp*⁺ cDNA probe to tissue sections of these embryos, we have found that the neural cells of the transformed segments accumulate *Antp*⁺ transcripts to a level characteristic of the normal mesothorax.

Drosophila neurogenesis involves the formation of a ventral nerve cord. On its initial establishment during early embryogenesis, the ventral cord is a composite of discrete, repeating ganglia¹⁰. The epidermis of each of the three thoracic and eight

abdominal segments of the embryo is innervated by a corresponding ganglion of the ventral cord. Figure 1a shows an autoradiograph of a tissue section through an 18-h wild-type embryo after hybridization to a tritium-labelled *Antp*⁺ cDNA probe. The same tissue section is shown in Fig. 1b after dark-field photomicroscopy. At this advanced stage of embryogenesis, condensation of the ventral nerve cord has resulted in the loss of overt segmentation. Nonetheless, from similar examination of younger embryos^{7,8}, it is clear that the highest accumulation of silver grains corresponds to the ganglion cells of the mesothoracic segment (arrow). It is possible that the strong hybridization signal includes some of the ganglion cells in the posterior portion of the prothoracic segment. Comparisons of tissue sections through different embryos of the same stage indicate that the orientation of these strongly labelled cells is roughly constant with respect to the proventriculus (pv), which therefore serves as an indicator of the mesothoracic ganglion. The other ganglia of the ventral cord display much weaker labelling.

The chromosomal deficiency *Df(3R)P9* deletes all known genes of the *bithorax* complex and embryos homozygous for this allele die during the terminal stages of embryogenesis. Inspection of the cuticle of such embryos indicates that the metathorax and the first seven abdominal segments acquire features characteristic of both the posterior portion of the normal prothoracic segment and the anterior portion of the mesothorax^{1,4,9}. Figure 2a shows an autoradiograph of an embryo homozygous for the *P9* deficiency after hybridization to the *Antp*⁺ cDNA probe. The corresponding dark-field photomicrograph of this tissue section is shown in Fig. 2b. As compared with a wild-type embryo of similar age (Fig. 1), a much larger proportion of the ventral ganglion cells is strongly labelled in the *P9* embryo. This labelling extends uniformly posteriorly from the ganglion cells of the mesothoracic segment through those of the first seven abdominal segments. As in wild-type embryos, the ventral ganglion cells of the anterior portion of the prothoracic segment and of the 8th abdominal segment show considerably less labelling at this stage.

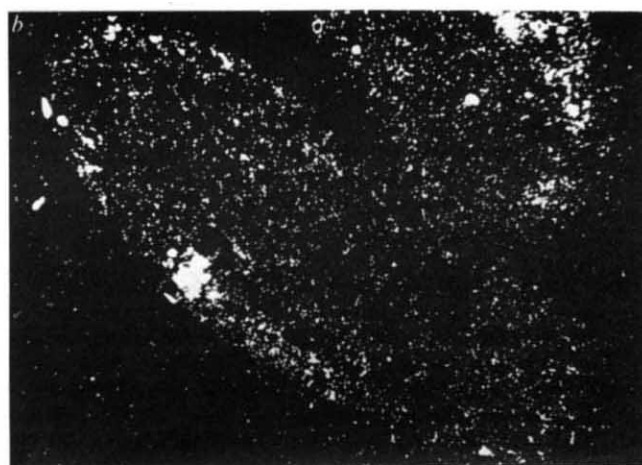
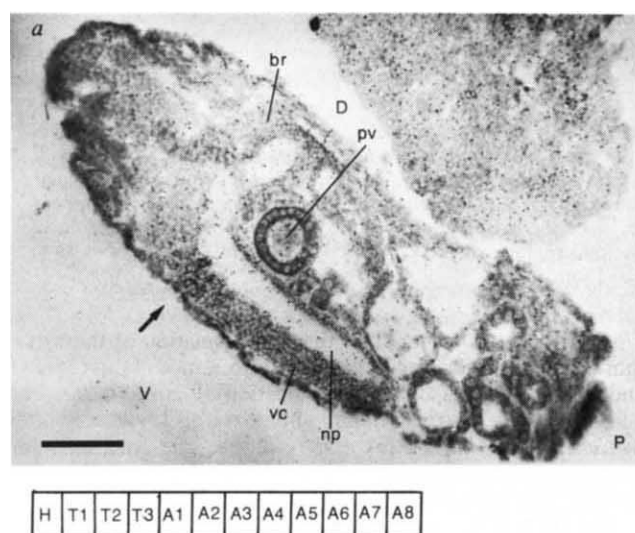


Fig. 1 Localization of *Antennapedia*⁺ (*Antp*⁺) transcripts in a sagittal section through an 18-h wild-type embryo. *a*, A bright-field photomicrograph of the embryo section; *b*, a dark-field photomicrograph of the same embryo section. The diagram in *a* is a schematic representation of the segmentation pattern of the embryonic cuticle and the nervous system. A, anterior aspect of the embryos; A1–A8, eight abdominal ganglia of the ventral nervous system; br, brain; D, dorsal aspect of the embryo; np, neuropile; P, posterior aspect of the embryo; pv, proventriculus; T1–T3, three thoracic ganglia of the ventral nervous system; V, ventral aspect of the embryo; vc, ventral cord. Scale bar, 0.1 mm. The section was hybridized to the 903 *Antp*⁺ cDNA probe and autoradiographed for 15 days. 903 is a pBR322 recombinant containing a 2.2-kilobase (kb) cDNA insert that is the reverse transcription product of poly(A)⁺ RNA extracted from 0–8 h wild-type embryos. This cDNA segment was shown to share homology with four non-contiguous chromosomal DNA sequences encompassing 100-kb cloned genomic DNA derived from the *Antp*⁺ locus⁶. The 903 recombinant was tritium-labelled and hybridized to frozen tissue sections of staged wild-type embryos as previously described⁷. The arrow indicates strong hybridization of the 903 cDNA probe to the mesothoracic ganglion cells.

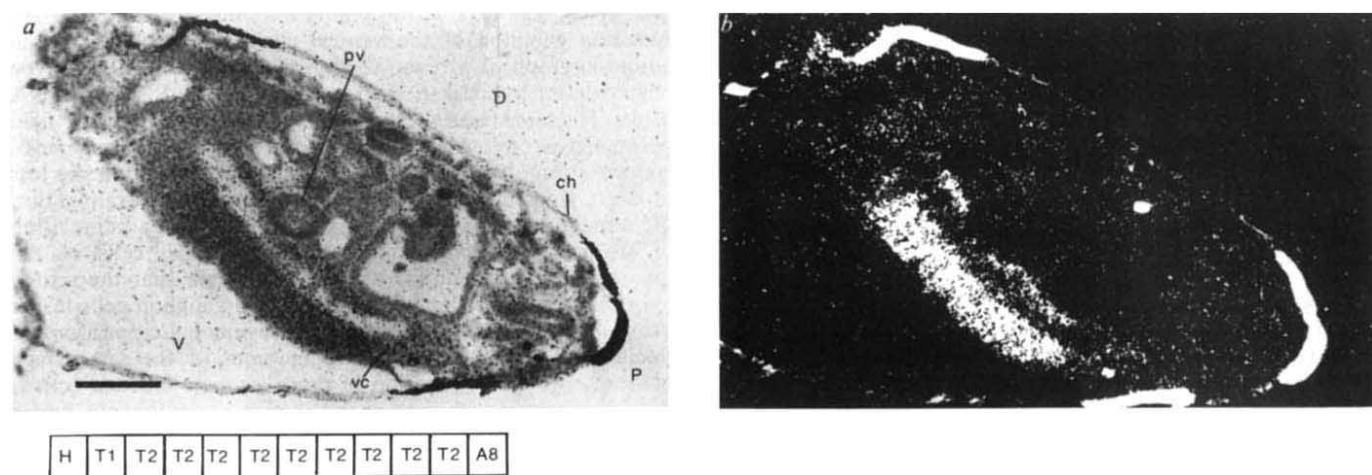


Fig. 2 Localization of *Antennapedia*⁺ (*Antp*⁺) transcripts in an embryo homozygous for the *P9* deficiency of the *bithorax* complex. A dark-field photomicrograph of the section shown in *a* is presented in *b*. The diagram in *a* is a schematic representation of the cuticular segmentation pattern of the embryos homozygous for the *P9* deficiency. Abbreviations as in Fig. 1 and ch, chorion shell. Scale bar, 0.1 mm. Embryos were collected from the mutant strain *Dp(3.3)P100/Df(3R)P9* (kindly provided by E. B. Lewis) for 4 h and the embryos were allowed to develop at 25 °C for an additional 26 h, so that all the viable embryos were able to hatch. The unhatched embryos were collected and sections were prepared as described previously⁷ except that the embryos were embedded without removal of the chorion shell and without prefixation in paraformaldehyde. *a* Shows a sagittal section through a 26-h embryo homozygous for the *P9* deficiency after hybridization to the 903 *Antp*⁺ cDNA probe and autoradiography for 24 days.

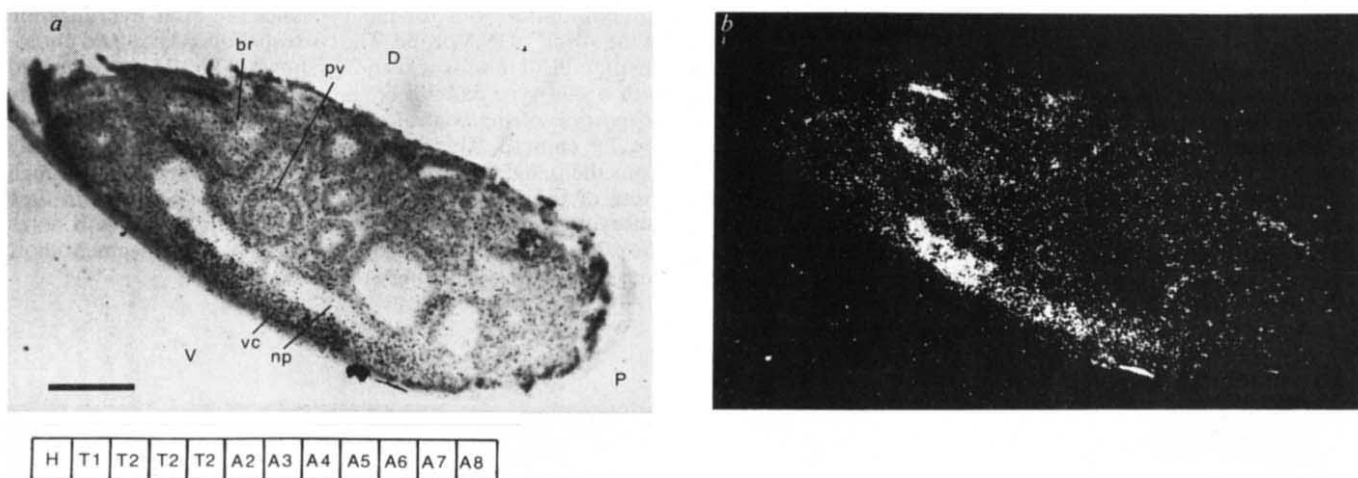


Fig. 3 Localization of *Antennapedia*⁺ (*Antp*⁺) transcripts in a homozygous *Ubx*¹⁰⁵ embryo. Embryos were collected from four winged flies heterozygous for *Ubx*¹⁰⁵ and two recessive *bithorax* alleles, *bx* and *pbx*. *a*, A sagittal section through a 20-h embryo homozygous for *Ubx*¹⁰⁵ after hybridization to the 903 *Antp*⁺ cDNA probe and autoradiography for 24 days; the diagram is a schematic representation of the cuticular segmentation pattern of embryos homozygous for *Ubx*¹⁰⁵. The accumulation of silver grains at the presumptive mouthhooks does not appear to be due to specific interaction of the probe DNA with a cognate RNA, since the same pattern was occasionally observed with control hybridization probes not derived from the *Antp*⁺ locus. *b*, Dark-field photomicrograph of the same section as in *a*. Abbreviations as in Fig. 1. Scale bar, 0.1 mm.

In addition to the homeotic transformation of the metathoracic and abdominal epidermis in *P9* embryos, it has been shown that the ganglia of these more posterior segments possess some of the morphological features characteristic of a normal thoracic ganglion¹¹. During the latter periods of normal embryogenesis the ganglion cells of the mesothorax possess substantially higher concentrations of *Antp*⁺ RNAs than do the ganglion cells of the other segments^{7,8}. Therefore, strong hybridization signals with the *Antp*⁺ cDNA probe serve to indicate the identity of mesothoracic ganglion cells. As a result, the strong labelling of the metathoracic and first seven abdominal ganglia of *P9* embryos is a further indication that, in addition to the epidermis, there is also a corresponding mesothoracic transformation of the neural tissue.

The distribution of *Antp*⁺ transcripts was also examined in embryos homozygous for the *Ubx*¹⁰⁵ allele of the *bithorax* gene complex. Such individuals die either during late larval development or during pupation. The terminal cuticular phenotype of

*Ubx*¹⁰⁵ homozygotes involves the transformation of the metathorax and first abdominal segment into a mesothorax (ref. 1 and E. B. Lewis, personal communication). Figure 3*a* shows an autoradiograph of a 20-h embryo homozygous for *Ubx*¹⁰⁵ after hybridization to the *Antp*⁺ cDNA probe. The corresponding dark-field photomicrograph is shown in Fig. 3*b*. As compared with the wild-type pattern shown in Fig. 1, a larger proportion of ventral ganglion cells display strong hybridization signals. The anterior-most labelled ganglion cells are located in a similar position with respect to the proventriculus as are the mesothoracic ganglion cells of a wild-type embryo of similar age (Fig. 1). However, in contrast to the wild-type hybridization pattern, an increased accumulation of silver grains is also observed in more posterior ganglion cells. Based on estimates of the number of ganglion cells within each segment of the ventral cord, the extent of the labelling is sufficient to encompass the metathoracic and first abdominal ganglia. Thus, in the absence of normal *Ubx*¹⁰⁵ function, there appears to be a trans-

formation of both the neural and epidermal tissue of the metathorax and first abdominal segment to the homologous tissue of the mesothorax.

The results presented here indicate that *bithorax* mutants which are wild type with respect to the *Antp*⁺ gene exhibit an altered pattern of *Antp*⁺ expression. In such individuals a close correlation has been established between the phenotypic transformation of the cuticular portion of the metathoracic and abdominal segments to a mesothorax and an augmented steady-state level of *Antp*⁺ RNA present in the ganglion cells of the affected segments. Evidence supporting a role for *Antp*⁺ products in the specification of the mesothorax had been previously obtained by examination of the pattern of *Antp*⁺ expression in wild-type embryos by *in situ* hybridization^{7,8}. Even during early periods of normal embryonic development the mesothoracic segment in the ventral nervous system is associated with relatively high levels of *Antp*⁺ RNAs⁸. The present study substantially extends this observation. In the absence of normal *bithorax* function, *Antp*⁺ RNAs accumulate in the progenitors of more posterior ventral ganglia where they might function to impose a mesothoracic developmental pathway.

During early periods of normal embryonic development, *Antp*⁺ transcripts are detected in the neural progenitors of each of the thoracic and abdominal ganglia. However, during subsequent development, the RNAs that are initially detected in the pro-, metathoracic and abdominal ganglia gradually diminish such that by mid-embryogenesis the mesothoracic ganglion cells contain the highest concentrations of *Antp*⁺ transcripts⁸. Once established, this segment-specific pattern of *Antp*⁺ expression is maintained through the remaining periods of embryonic development and subsequent larval development. It has been proposed that expression of the *bithorax* gene complex is confined to the metathoracic and abdominal segments¹. Consistent with this proposal is the recent demonstration that *Ultra-bithorax*⁺ (*Ubx*⁺) transcripts are distributed along the ventral cord of developing wild-type embryos as observed for *Antp*⁺ transcripts, except that by late embryogenesis the ganglion cells of parts of the metathorax and the first abdominal segment possess the highest concentrations of *Ubx*⁺ RNAs¹². *Antp*⁺ transcripts might be prevented from reaching high steady-state levels in the posterior ganglia of wild-type embryos by the *bithorax* products which are also present in these cells. In the mesothorax, *Antp*⁺ RNAs stably accumulate since *bithorax* products are largely absent from this segment. Thus, one possible explanation for the increased levels of *Antp*⁺ transcripts in the metathoracic and abdominal ganglia of P9 embryos is the absence of *bithorax* products from these positions. This interpretation of the results agrees with previous genetic findings which suggest a direct or indirect regulatory influence of products from the *bithorax* complex on the proper expression of genes in the *Antp* complex⁵.

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Activation of extra copies of genes coding for nitrogenase in *Rhodospseudomonas capsulata*

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Biological nitrogen fixation requires the nitrogenase enzyme complex, ATP, and a strong reductant¹. *Klebsiella pneumoniae* contains 15 linked nitrogen fixation (*nif*) genes^{2,3}, three of which, *nifH*, *nifD* and *nifK* have been sufficiently conserved in evolution that cloned *K. pneumoniae* *nifHDK* DNA⁴ will hybridize to DNA sequences from every nitrogen-fixing bacterium examined to date, including the purple, non-sulphur bacterium *Rhodospseudomonas capsulata*⁵, in which one complete *nifHDK* operon has been mapped⁶. Using cloned *K. pneumoniae* *nifHDK* DNA we report here that *R. capsulata* contains multiple copies of the genes for nitrogenase components. Two regions containing sequences homologous to all three *nif* structural genes have been identified, and mutations in one region produced a Nif⁺ phenotype. Nif⁺ pseudorevertants were derived from these mutants, some of which retained the original mutation suggesting that some of the extra *nif* gene sequences can be functionally activated.

Hybridization of pSA30 to a *Hind*III digest of total DNA from *R. capsulata* shows two strong bands of 11.8 kilobasepairs (kbp) and 4.9 kbp, as well as additional bands of lower intensity (and presumably lower homology) (Fig. 1, lane a). A *nifH* gene-specific probe hybridized only to the 11.8 and 4.9-kbp fragments (lane b), while *nifD* and *nifK* probes annealed to several additional bands including two at 3.6 and 1.9 kbp (lanes c, d). The 11.8-kbp *Hind*III fragment contains the functional *nifHDK* operon previously mapped⁶, referred to as 'copy I' (Fig. 3, for strain SB1003). The results in Fig. 1 show that there is at least one additional *nifH*-related sequence in the 4.9-kbp *Hind*III fragment and several additional *nifD*- and *nifK*-related sequences. Other related sequences appear to be present on two larger fragments as well.

Multiple copies of *nif* gene-related sequences are also seen in the *Eco*RI digest, particularly with the single gene probes. The two bands that are identified by pSA30 at 7.6 and 5.0 kbp (lane e) derive from copy I; an *Eco*RI site is near the C-terminal end of the *nifD* gene (Fig. 3). Three additional bands, 11.0, 6.5 and 6.0 kbp, are identified by all three single gene probes (lanes f-h), enabling us to conclude that *R. capsulata* contains at least four copies of sequences related to the structural genes for nitrogenase components.

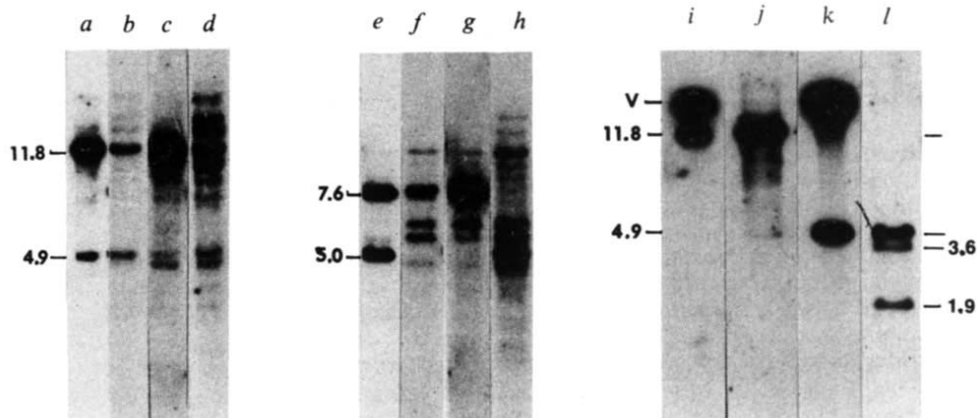
To clone the *nif*-related sequences for detailed study, we first isolated a fragment of copy I DNA by screening a library of recombinant λ containing *Eco*RI fragments of *R. capsulata* DNA in λ gt7-*ara*6 (R. W. Davis, unpublished). One such recombinant phage contained the 7.6-kbp *Eco*RI fragment from copy I, which contains all of *nifH* and most of *nifD* (Fig. 1, lanes e-h, and Fig. 3). That fragment was purified and used to probe a cosmid library of *Hind*III fragments (Fig. 1 legend). Two cosmids were found to contain related sequences. Plasmid p103 contains the 11.8-kbp *Hind*III fragment and an additional 32 kbp of adjacent chromosomal DNA, while plasmid p108 contains the 4.9-kbp *Hind*III fragment and an additional 40 kbp of chromosomal DNA. The two cosmids have no restriction fragments in common.

Further study of the relationships among the *nif*-related sequences was facilitated by subcloning the 11.8- and 4.9-kbp *Hind*III fragments from p103 and p108, respectively, into the mobilizable vector pRK292 (ref. 7). The results of hybridizing each of these plasmids back to total chromosomal DNA of *R. capsulata* are shown in Fig. 1, lanes j and l. These results indicate that the extra copies are more closely related to each other than any of them is to copy I.

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Fig. 1 Multiple copies of *nif* gene-related sequences in *R. capsulata* DNA revealed by Southern hybridization with heterologous probes. Lanes a–d contained *R. capsulata* DNA digested with *Hind*III; lanes e–h the same DNA digested with *Eco*RI. Radioactive probes contained *Klebsiella nifHDK* DNA (lanes a, e), *Klebsiella nifH* (lanes b, f), *Anabaena nifD* (lanes c, g) and *Klebsiella nifK* (lanes d, h). The 11.8-kbp *Hind*III fragment and two major *Eco*RI fragments of 7.6 and 5.0 kbp are derived from copy I of the *nifHDK* operon (see map in Fig. 3). The 11.8-kbp and 4.9-kbp *Hind*III fragments were isolated from a cosmid library and subcloned in pRK292 to yield plasmids pRPA5 and pRPA7, respectively. pRPA5 was used as probe in Southern hybridization to *Hind*III cut pRPA5 (lane i) or total *R. capsulata* DNA (lane j). pRPA7 was the probe with pRPA7 (lane k) or total *R. capsulata* (lane l). V indicates the vector DNA band. The extra copy probe, pRPA7, identifies copies which were less visible in lanes c and d.



Methods: Total DNA from *R. capsulata* was isolated by the method of Bendich *et al.*¹⁸. Approximately 2 µg were digested with *Hind*III or *Eco*RI and the resulting fragments were separated on 0.7% agarose gels. DNA was transferred to nitrocellulose by the method of Southern¹⁹ and annealed with $1-2 \times 10^6$ d.p.m. of ³²P-labelled nick-translated probe. Hybridization was for 20 h at 65 °C in 5×SSPE, (0.15 M NaCl, 0.01 M NaH₂PO₄, 1 mM Na₂EDTA, pH 7.0) followed by three 45-min washes in 3×SSPE. The *Klebsiella nifHDK* probe pSA30 has been described⁴. The internal *nifH* probe was prepared by subcloning an *Eco*RI–*Bgl*II fragment⁴ from pSA30 into pBR322 cut with *Eco*RI and *Bam*HI. The *nifD* probe was pAn256, which contains most of the *nifD* gene of *Anabaena* 7120¹⁵. The internal *nifK* probe was prepared by subcloning a *Sall* fragment⁴ from pSA30 into the *Sall* site of pBR322. The cosmid library of *R. capsulata* DNA was prepared from a size-fractionated *Hind*III partial digest ligated into the cosmid vector pDPT5Cm (ref. 20) and maintained in *E. coli* HB101. Screening the library with the 7.6-kbp *Eco*RI fragment (lane e), which had been isolated from a λ phage library, yielded cosmids p103 and p108. Each cosmid contained over 40 kbp *R. capsulata* DNA. The 11.8-kbp *Hind*III fragment (copy I) was subcloned from p103 into the mobilizable plasmid pRK292 (ref. 7) to yield pRPA5 (lanes i, j). The 4.9-kbp fragment was subcloned from p108 into pRK292 to yield pRPA7 (lanes k, l).

We next wished to study the functional properties of the cloned *nif* gene-containing fragments. For this purpose, we designed a method for site-specific mutagenesis of *R. capsulata* using the gene transfer agent (GTA)⁸, a defective transducing phage that packages 4.6-kbp of linear, double-stranded chromosomal DNA fragments⁹, to make insertions or deletions. The method is illustrated in Fig. 2. Briefly, a 2.4-kbp *Xho*I fragment (the 'cartridge', carrying the gene for kanamycin resistance from Tn5 (ref. 10) (which is expressed in *R. capsulata*) is ligated *in vitro* into a *Xho*I, *Sall* or *Ava*I site in a cloned target fragment. The plasmid so constructed is used to transform *Escherichia coli* and then mobilized into a GTA-overproducing strain of *R. capsulata*. Finally, GTA from this strain is used to transfer Km^r to a recipient *R. capsulata*. Km^r transductants are produced by a double recombination event, one in each of the sequences flanking the Km^r gene. Although this method can only be used in *R. capsulata*, it has the advantages over previously described procedures¹¹ that it does not depend on transposition to generate insertions and it does not leave a plasmid behind after the insertion or deletion is created in the recipient chromosome.

The results of hybridization to *Hind*III digests of total DNA from wild-type *R. capsulata* and from strain 1051, in which the Km^r cartridge has been inserted in the *Sall* site of the *nifH* gene of copy I, are shown in Fig. 2, e and f, respectively. The probe in this case was pRPA5, which contains the cloned 11.8-kbp fragment. The 2.4-kbp Km^r cartridge contains a single *Hind*III site (Fig. 2), so inserting it into the *nifH* gene introduces a new *Hind*III site into the 11.8-kbp fragment. The two fragments produced, 9.9 and 4.3 kbp, correspond to the sum of 11.8 and 2.4 kbp.

Maps of one insertion and two deletions produced in copy I by this method are shown in Fig. 3, with details of the strain constructions. Southern hybridizations confirming the map positions of the insertions and deletions are shown in Fig. 4. The hybridization of DNA from strain 1051 was described above. In strain DN51, the 750-bp *Xho*I fragment in *nifD* was deleted and replaced with the 2.4-kbp Km^r cartridge. Thus, the 11.8-kbp *Hind*III fragment is replaced by two new fragments (Fig. 4, lane d) that total 13.4 kbp, while the 7.6-kbp *Eco*RI fragment is

replaced by one of $7.6 + 2.4 - 0.75 = 9.25$ kbp (Fig. 4, lane h). The latter result can be compared with that for strain 1051 (lane f) in which the insertion was made without deletion; the new band in that case is 10.0 kbp. Finally, in strain 306, the large *Ava*I fragment entirely within the 7.6-kbp *Eco*RI fragment was deleted and replaced with the Km^r cartridge. In Fig. 4, lane c, the two new *Hind*III fragments total 7.2-kbp and in lane g the new *Eco*RI fragment is 3.0 kbp, consistent with the map shown in Fig. 3. The blots in Figs 2 and 4 also show that only the target sequence is affected; the extra copies are unaltered in strains in which the Km^r marker was inserted in copy I. All of the strains in which the Km^r marker was inserted in copy I are Nif⁺, indicating that, in the conditions used in the laboratory, only copy I *nifHDK* genes are functional. This conclusion has also been reached on the basis of complementation of independently isolated Nif[−] point mutants by the cloned copy I fragment⁶.

The insertions and deletions created in copy I *nif* genes should not revert. However, Nif⁺ pseudorevertants were obtained from both types of mutations. We obtained Km^r, Nif⁺ strains from each of the mutants shown in Fig. 3. Such strains grew slowly in nitrogen-free media and had about 10% of the wild-type acetylene reduction activity. These strains retained the Km^r cartridge in the copy I genes, shown by hybridization to total DNA prepared from the revertants (data not shown). Some Nif⁺ revertants of strain 1051 were found to be Km^s. These strains had wild-type level nitrogenase activity and their DNA showed restoration of the wild-type restriction pattern (*Hind*III and *Eco*RI) in the copy I genes. We think that, in these strains, extra copy *nifH* sequences were used to convert (delete) the inserted cartridge at copy I. Precise excision of the cartridge is less likely because the only region of homology at the ends of the insert is the *Xho*I site.

These results indicate that sequences homologous to all three structural genes for nitrogenase are present in several copies in the *R. capsulata* genome, but differences in the restriction pattern and in the intensity of hybridization with the specific probes indicate that the multiple sequences are not identical. Do the extra copies of the *nif* genes have any physiological meaning? At present we know too little about them to offer an

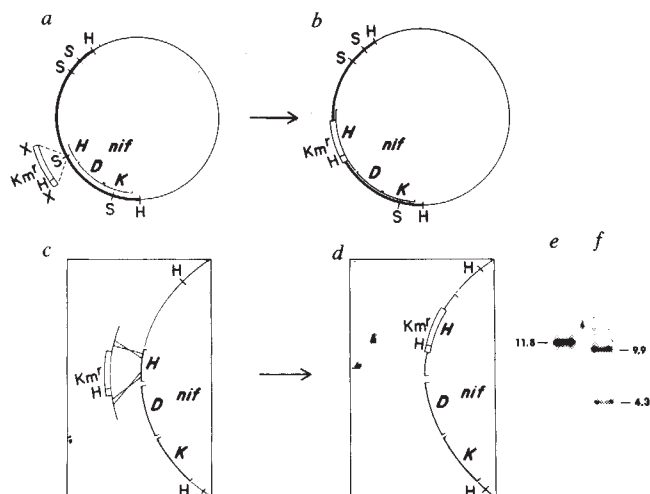


Fig. 2 Site-directed mutagenesis of *Rhodospseudomonas capsulata* with a kanamycin resistance gene cartridge. **a**, Plasmid pRPA5 DNA (Tet^r) was digested with *SalI* in the presence of ethidium bromide²¹ to generate one cut per molecule. A 2.4-kbp *XhoI* fragment carrying Km^r from Tn5 was ligated in and the resulting plasmid population was used to transform *E. coli* HB101, selecting for tetracycline and kanamycin resistance, to yield **b**. Plasmid DNA from individual clones was extracted and digested with *HindIII* and *EcoRI*. The Km^r cartridge contains one *HindIII* site and no *EcoRI* sites, so the original 11.8-kbp fragment is replaced in the insertions by two *HindIII* segments (9.9 and 4.3 kbp for the insert in *nifH* oriented as shown), and the 7.6-kbp *EcoRI* fragment containing *nifH* and most of *nifD* (see map in Fig. 3) is replaced by a 10-kbp fragment. Insertions in several *SalI* sites were obtained. The insert in the *nifH* site was identified by hybridizing the *HindIII* cut plasmid fragments with ³²P-labelled pSA30. Since this probe is homologous only to the *nifHDK* region and both *HindIII* fragments showed homology, the insertion has to be in the *nifH* *SalI* site shown. **b**, This plasmid was then mobilized into *R. capsulata* Y262, a gene transfer agent (GTA) overproducer⁹, using the helper plasmid pRK2013. The conjugational cross was done on RCVB minimal medium, selecting for tetracycline resistance (5 µg ml⁻¹). *E. coli* HB101 does not grow in this medium. GTA was prepared from these cells⁹ and used to transduce wild-type *R. capsulata* SB1003, selecting for Km^r. Since GTA transduces linear, double-stranded segments of DNA 4.6 kbp in size, any fragment containing genomic DNA on both sides of the Km^r cartridge can recombine with the target sequences in the chromosome of the recipient cell (**c**) generating a Km^r mutant (strain 1051) (**d**). Total genomic DNA from strains SB1003 (**e**) and 1051 (**f**) was digested with *HindIII*, and the fragments separated in a 0.7% agarose gel, blotted onto nitrocellulose and hybridized with ³²P-labelled pRPA5 DNA. The *HindIII* site is 750 bp from one end of the *XhoI* Km^r fragment. The *SalI* site is 3.55 kbp from the right end of the 11.8-kbp *HindIII* fragment of wild-type DNA. Thus, the wild-type 11.8-kbp band in **e** has been replaced by two fragments (9.9 and 4.3 kbp) in strain 1051 (**f**), corresponding to 8.25 + 1.65 kbp and 0.75 + 3.55 kbp, respectively. This method may also be used to generate deletions (Figs 3, 4) by removing a restriction fragment at the time of cartridge insertion *in vitro*.

answer. We would like, however, to discuss two possibilities: first, that the extra copies are pseudogenes and, second, that they are activated in conditions different from those used here.

The first model implies that the extra sequences are permanently silent, which might be expected for evolutionary remnants from a process of lateral diffusion of *nif* genes in nature¹². The process of activation to produce the pseudorevertants would create a promoter in front of the coding region(s) of one or more copies, or it would consist of mutations which 'repair' the DNA sequence so as to produce active products, or both.

The second possibility is that the extra copies are functional genes which become activated in growth conditions different from those used here. This model would predict that the nitrogenase encoded by the extra copies has properties (such as ammonia switch-off, substrate affinity, cofactor requirements

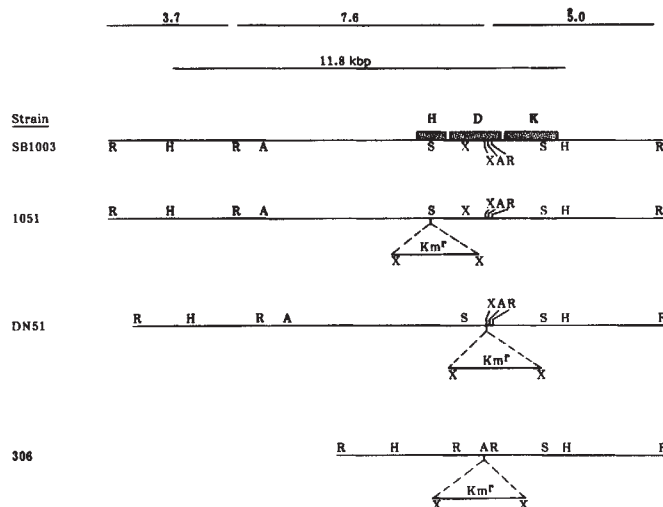


Fig. 3 Physical map of the copy I *nifHDK* region in different strains of *R. capsulata*. Lines at the top of the figure indicate the *EcoRI* and *HindIII* fragments, labelled in kbp, in wild-type *R. capsulata*, that hybridize with pRPA5. The *EcoRI* 7.6-kbp fragment contains the *nifH* gene and most of the *nifD* gene (see Fig. 1 and ref. 6). The 5.0-kbp *EcoRI* fragment contains the *nifK* gene. Location of genes and sites for *EcoRI*(R), *HindIII*(H), *SalI*(S), *AvaI*(A) and *XhoI*(X) are shown for the wild-type strain (SB1003). Strain 1051 carries a Km^r cartridge insertion in the *nifH* *SalI* site. DN51 has a 750-bp deletion of the *XhoI* segment in *nifD*. Strain 306 has a 7.0-kbp deletion between *AvaI* sites within the 7.6-kbp *EcoRI* fragment. The *HindIII* site in the Km^r cartridge is not shown in this figure.

Methods: Construction of strain 1051 was explained in Fig. 2 legend. To construct strain DN51, the *SalI* fragment of SB1003 containing sequences from *nifH* to *nifK* was subcloned into pBR322, and then digested with *XhoI* (which does not cut pBR322). To the resulting DNA we ligated the Km^r cartridge, and then transformed *E. coli* HB101, selecting for Tet^r Km^r. The resulting plasmid was mobilized by conjugation into *R. capsulata* Y262 with the helper plasmid pDPT51, selecting for Km^r. GTA was prepared and used to transduce Km^r to SB1003. Substitution of the target sequence by the segment carrying the Km^r cartridge results in the insertion of the cartridge and consequent deletion of the *XhoI* segment. To construct strain 306, we carried out a partial digestion of plasmid pRPA5 with *AvaI*. After inactivation of the enzyme, this DNA was ligated to the Km^r cartridge. Several *AvaI* sites are present in the 11.8-kbp *HindIII* fragment (not shown), so several deletions were obtained. Strain 306 is shown as an example. This strain grows normally in minimal medium, indicating that no essential genes were deleted. Its Nif⁻ phenotype is complemented by pRPA8, which contains only the copy I *nifHDK* operon⁷, so there are no other *nif* genes deleted in strain 306.

and regulation) different from copy I nitrogenase, and that the cell has the ability selectively to induce different copies according to varying environmental conditions. Pseudorevertants in this case would be mutants in the activation control mechanism. *R. capsulata* can grow and fix nitrogen anaerobically in the dark, provided a strong oxidant (such as dimethyl sulphoxide or trimethylamine oxide) is present in the medium¹³. However, these compounds are not present in natural environments in a concentration high enough to sustain growth. *R. capsulata* is constantly challenged by changing environmental factors, including interactions with other microorganisms, and it might be very difficult or impossible to reproduce these situations in the laboratory. A variation of this model is that the extra copies are actually silent, but a controlled gene rearrangement can bring genes or segments of genes to the copy I region, resulting in the expression of genes whose products have properties different from those of copy I. The phenotypic reversion of strain 1051 to Nif⁺, Km^s cells could be the consequence of such a process. These possibilities can be distinguished by sequencing and by characterization of the *nif* gene products in the pseudorevertants.

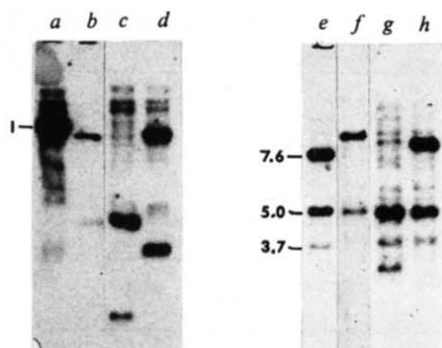


Fig. 4 Characterization of insertion and deletion mutations by Southern hybridization. Total DNA from the following strains was digested with *Hind*III (lanes a-d) or *Eco*RI (lanes e-h): SB1003, lanes a and e; 1051, lanes b and f; 306, lanes c and g; DN51, lanes d and h. The probe in each case was pRPA5, containing copy I *nifHDK* sequences. Strain 1051 contains the 2.4-kbp *Km*^r cartridge inserted in *nifH* of copy I (Fig. 3). The cartridge contains a *Hind*III site, leading to the two bands seen in lane b of 9.9 and 4.3 kbp. Lane f shows the 7.6-kbp *Eco*RI fragment increased to 10 kbp by the insert. Strain 306 contains two *Hind*III fragments of 4.4 and 2.8 kbp, while the 7.6-kbp *Eco*RI fragment is replaced by one of 3.0 kbp. These changes result from a deletion of 7 kbp and insertion of the 2.4-kbp *Km*^r cartridge (Fig. 3). Strain DN51 shows two *Hind*III fragments of 10.1 and 3.3 kbp, while the 7.6-kbp *Eco*RI fragment is increased to 9.25 kbp. These changes result from a 750-bp deletion and insertion of the *Km*^r cartridge (Fig. 3).

Methods: Pseudorevertants were isolated as follows: The *Km*^r *Nif*⁻ strains were grown overnight aerobically in RCVB minimal medium at 35 °C²². 0.1 ml of these cultures was inoculated into 10 ml of RCVB-NF (nitrogen-free) medium, and grown overnight aerobically at 35 °C to exhaust nitrogen completely. Cells were washed twice with RCVB-NF and approximately 10⁹ cells were placed in a 10-ml screwcap tube totally filled with RCVB-NF medium. These cultures were incubated until turbidity was observed (1–2 weeks). Cells were then streaked on RCVB-NF plates and incubated until 2-mm diameter colonies formed (3–4 days). The *Km* resistance phenotype was tested by replica plating onto RCVB and RCVB-*Km*. Strain 1051 gave two kinds of pseudorevertants: *Km*^r (R1051r) and *Km*^r (R1051s). Only *Km*^r *Nif*⁺ revertants were isolated from the deletion strains. To test nitrogenase activity, cells were grown overnight photosynthetically in RCVB, washed three times with RCVB-NF, resuspended in RCVB-NF and incubated photosynthetically for 8 h before determining acetylene reduction activity²³.

This work adds one more example of bacterial gene families. *Rhizobium phaseoli* has reiterated *nif* sequences¹⁴ and the cyanobacteria *Anabaena*¹⁵ and *Calothrix*¹⁶ have more than one copy of the *nifH* gene. Extra copies of structural genes other than *nif*, of which only one is normally expressed, have been described in other bacterial species. There are two non-identical copies of the spore coat protein gene of *Myxococcus xanthus*¹⁷, separated by a 1.2-kbp spacer. Only the downstream gene that codes precisely for the sequenced coat protein is expressed. A similar situation was found in *Caulobacter crescentus*: of at least three genes that could code for flagellin, the major flagellar protein, only one is normally expressed (N. Agabian, personal communication).

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Transposition of Tn554 does not generate a target duplication

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Transposable elements from prokaryotic and eukaryotic organisms are discrete DNA segments bounded by inverted or directly repeated sequences that insert into non-homologous DNA in a reaction that is independent of the general recombination functions of the host. The mechanisms proposed generally involve a staggered double-stranded scission of the target DNA, ligation to the nicked ends of the transposable element, and replication of the element, resulting in the generation of a directly repeated oligonucleotide target sequence flanking the new copy of the element^{1–3}. Most transposons have a relatively low degree of target site specificity coupled with a low insertion frequency. Tn554, a *Staphylococcus aureus* transposon which specifies resistances to erythromycin and spectinomycin, displays an unusually high degree of insertion specificity. Tn554 transposes with high efficiency to a unique ('primary') site in the *S. aureus* chromosome^{4,5} and only rarely (<10⁻⁶ per transductant) to other, secondary sites⁶. We report here the nucleotide sequences surrounding the junctions of Tn554 in three independent 'primary' insertions and two 'secondary' insertions of the transposon. Two unusual features are revealed: first, the termini of Tn554 contain neither inverted nor directly repeated sequences. Second, transposition of Tn554 does not generate the short direct repeats of the target DNA that are characteristic of other transposable elements. These results suggest that the mechanism of Tn554 insertion may be significantly different from that of other transposons.

Previous blot hybridization experiments (ref. 5 and unpublished data) indicated that insertion of Tn554, in each of 25 cases examined, occurred in the same location in the *S. aureus* chromosome, within the limits of resolution of this method. To determine the insertion specificity at the nucleotide level, the sequences of several independent transpositions of Tn554 into the chromosomes were determined. The following strategy was used to obtain multiple, independent insertions of Tn554 in the primary target. The transposon was first cloned from the *S. aureus* chromosome into *Escherichia coli* (Fig. 1). The flanking chromosomal sequences contained on this plasmid were then used as a probe to screen for a recombinant plasmid containing the target region from a Tn554-negative strain, RN450. The chromosomal insert was then recloned into a *S. aureus* vector (pRN8057) and this construct, pEM9605, was used as a target for transposition of Tn554. This method was used effectively

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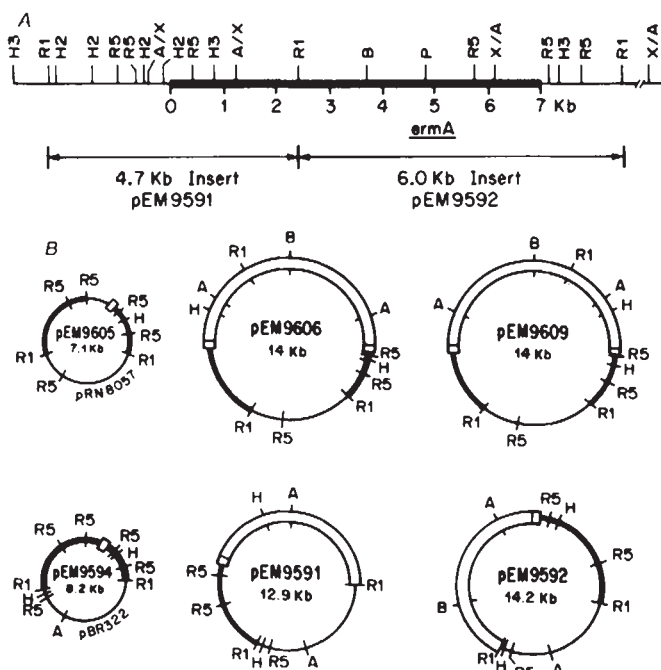


Fig. 1 **A**, Restriction map of Tn554 and flanking chromosomal DNA. Thick line, Tn554; thin lines, flanking DNA. Enzymes are: A, *Ava*I; B, *Bgl*III; H2, *Hpa*II; H3, *Hind*III; R1, *Eco*RI; R5, *Eco*R5; P, *Pst*I; X, *Xho*I. **B**, Restriction maps of Tn554-containing clones. Clones containing the primary (chromosomal) site sequences were obtained as follows: high molecular weight DNA from *S. aureus* strain RN2864 (Tn554⁺) was digested with *Eco*RI and the cleaved DNA fractionated on a 10–30% neutral sucrose gradient. Fractions were collected and aliquots loaded onto a 0.7% agarose gel; those containing DNA in the range of 3–7 kb were pooled, the DNA was ethanol-precipitated, and ligated to *Eco*RI-cleaved, alkaline phosphatase-treated pBR322. This preparation was used to transform *E. coli* HB101 with selection for carbenicillin resistance. Transformants were transferred to nitrocellulose filters for colony hybridization²⁰, using as a probe a 4.9-kb *Ava*I fragment from pRN4177, a secondary-site insertion of Tn554⁶. As Tn554 contains a single *Eco*RI site, clones were isolated that contained either the left (pEM9591) or right (pEM9592) half of Tn554, as indicated above. The latter carries the *Em*^r locus, which is expressed in *E. coli*. A clone containing the primary chromosomal insertion site (but lacking Tn554) was obtained similarly, using *Eco*RI-cleaved DNA from RN450, a standard laboratory host which does not contain Tn554. Sucrose gradient fractions containing DNA of the predicted size of the *Eco*RI fragment (3.8 kb) were used for the ligation. The probe for the colony hybridization was the *Eco*RI insert from pEM9592, containing the right half of Tn554 and 1.5 kb of flanking chromosomal DNA. Positive colonies contained a plasmid with a 3.8-kb *Eco*RI insert which matched the predicted restriction map of the target region. The 3.8-kb insert was subsequently recloned onto pRN8057, a temperature-sensitive derivative of the tetracycline resistance plasmid pT181, and transferred back to *S. aureus* (namely pRN9605).

for transposon Tn7, which exhibits an insertion preference for the *E. coli* chromosome that is similar to that of Tn554^{7,8}.

Insertions into pEM9605 were obtained by transduction, using as donors a strain having any one of five penicillinase plasmids containing Tn554 in a secondary site. We have previously observed that Tn554 transpositions obtained in this manner are usually associated with loss of the donor plasmid⁶. Erythromycin-resistant (*Em*^r) transductants were therefore screened for the absence of the donor plasmid markers (cadmium and penicillin resistances) and their plasmid contents analysed on agarose gels. Transductants fell into three classes: those containing only the 7.1-kilobase (kb) target plasmid pEM9605 and a chromosomally inserted Tn554, those containing only a 14-kb plasmid resulting from the transposition of Tn554 into pEM9605, and those containing plasmids of various

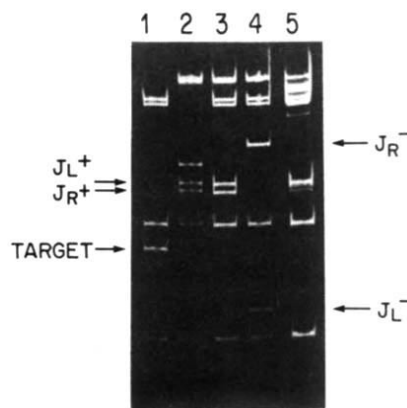


Fig. 2 Restriction analysis of transpositions into pEM9605. Plasmid DNA was prepared as described previously²¹, cleaved with *Eco*RV and fractionated on a 5% polyacrylamide gel. Lane 1, pEM9605 (target); lane 2, pEM9595; lane 3, pEM9606 (pRN4176 → pEM9605, +); lane 4, pEM9609 (pRN4177 → pEM9605, -); lane 5, pEM9613, a deleted derivative from the cross $\phi 11::Tn554 \rightarrow pEM9605$. *Eco*RV junction fragments J_L^+ and J_R^+ , for the (+) insertion, are approximately 1,200 and 980 bp, respectively; for the (-) insertions they are 402 bp (J_L^-) and approximately 1,800 bp (J_R^-); the *Eco*RV restriction fragment containing the Tn554 target site is ~550 bp long. (Restriction maps of pEM9605, pEM9606 and pEM9609 are shown in Fig. 1B.)

intermediate sizes, representing deleted derivatives of pEM9605::Tn554 insertions. As reported for Tn7 (ref. 7), transposition into the cloned site does not seem to be as efficient as insertion into the chromosome itself.

Restriction analysis of plasmid DNA from these transductants indicated that Tn554 inserted into the target fragment present in pEM9605 in the same location into which it inserts in the chromosome (Fig. 2). However, in contrast to the chromosomal insertions, which are orientation and site-specific, insertions into pEM9605 were recovered in both orientations. Recombination between flanking homologous sequences cannot account for the observed orientation specificity of the chromosomal insertions, as this specificity is maintained in the absence of flanking homology (that is, from non-homologous donors) as well as in the *recA* host⁵.

Most of the plasmids containing Tn554 insertions in the same orientation as in the chromosome (designated '+') were structurally unstable and could be detected only in the primary transductant colonies; these gave rise to deleted derivatives that had preferentially lost most of Tn554. Insertions into pEM9605 in the opposite orientation (designated '-') and some of the (+) orientation insertions, were stable. It is likely that insertions occurred with equal frequency in both orientations, but that only one class is recovered. Differences between the sequences present on the chromosome beyond the cloned region, and on the vector pRN8057, might account for the instability of the (+) transpositions in pEM9605. Note that the 3.8-kb insert of pEM9605 could itself be cloned in only one orientation into pRN8057; repeated efforts to reverse the orientation of the target fragment failed. In addition, experiments using a host strain carrying a chromosomal deletion of the primary insertion site suggest that the instability may be a consequence of recombinational events between the chromosome and the cloned chromosomal sequences (M. Bastos and E.M., unpublished data).

The nucleotide sequences of the junctions of several independent Tn554 insertions are shown in Fig. 3. Two unusual features of the Tn554 junctions are apparent. First, the ends of Tn554 lack any direct or inverted repeat sequences. Second, insertion of Tn554 does not generate a repeat of the target DNA. These results contrast with all other known transposable elements except for bacteriophage Mu, which also has asymmetric ends, but which generates a 5-base pair (bp) duplication⁹. Tn7, although similar to Tn554 in its insertion specificity with respect

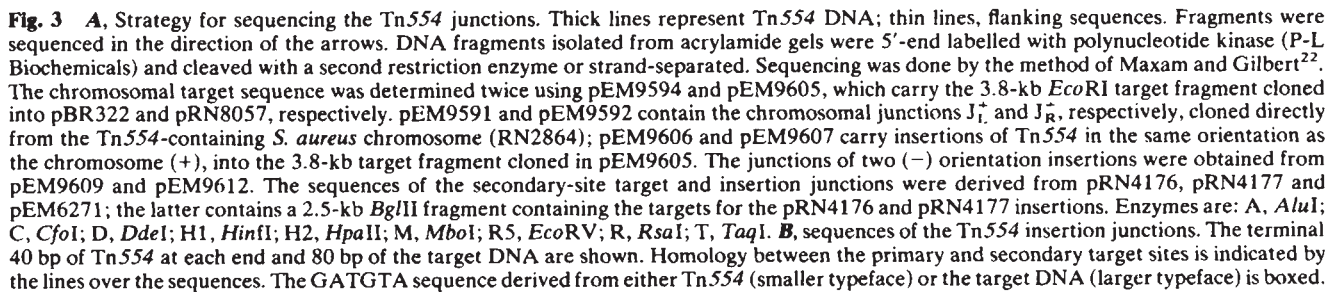


Fig. 3 **A**, Strategy for sequencing the Tn554 junctions. Thick lines represent Tn554 DNA; thin lines, flanking sequences. Fragments were sequenced in the direction of the arrows. DNA fragments isolated from acrylamide gels were 5'-end labelled with polynucleotide kinase (P-L Biochemicals) and cleaved with a second restriction enzyme or strand-separated. Sequencing was done by the method of Maxam and Gilbert²². The chromosomal target sequence was determined twice using pEM9594 and pEM9605, which carry the 3.8-kb *Eco*RI target fragment cloned into pBR322 and pRN8057, respectively. pEM9591 and pEM9592 contain the chromosomal junctions J_L⁺ and J_R⁺, respectively, cloned directly from the Tn554-containing *S. aureus* chromosome (RN2864); pEM9606 and pEM9607 carry insertions of Tn554 in the same orientation as the chromosome (+), into the 3.8-kb target fragment cloned in pEM9605. The junctions of two (-) orientation insertions were obtained from pEM9609 and pEM9612. The sequences of the secondary-site target and insertion junctions were derived from pRN4176, pRN4177 and pEM6271; the latter contains a 2.5-kb *Bgl*II fragment containing the targets for the pRN4176 and pRN4177 insertions. Enzymes are: A, *Alu*I; C, *Cfo*I; D, *Dde*I; H1, *Hinf*I; H2, *Hpa*II; M, *Mbo*I; R5, *Eco*RV; R, *Rsa*I; T, *Taq*I. **B**, sequences of the Tn554 insertion junctions. The terminal 40 bp of Tn554 at each end and 80 bp of the target DNA are shown. Homology between the primary and secondary target sites is indicated by the lines over the sequences. The GATGTA sequence derived from either Tn554 (smaller typeface) or the target DNA (larger typeface) is boxed.

Earlier data concerning excision of Tn554 (ref. 4) had led to the suggestion that Tn554 inserts and excises like bacteriophage λ ¹², using a free circular intermediate. The presence of the common GATGTA sequence at both the right end of Tn554 and at the site of insertion, and the absence of a target duplication, are consistent with this hypothesis. However, if Tn554 inserted via recombination between the 'common core' sequence GATGTA, integration would of necessity be orientation-specific and would not account for the insertions in the (-) orientation

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Sedimentation in Loch Earn and Loch Lubnaig, Scotland

In their paper about sedimentary features associated with slumping in Loch Earn and Loch Lubnaig, McManus and Duck¹ indicate that there is no prior record of the use of sonar devices in lakes for the investigation of "subaqueous landforms". This is a curious statement given our² earlier reported work in their own field area and the extensive surveys of the Canadian Centre for Inland Waters in for example, the Great Lakes (see ref. 3), where acoustic techniques have become standard for mapping both lakebed and sub-bottom landforms, whose recognition is regarded as a normal prerequisite for any engineering undertaking.

While McManus and Duck¹ recognize that their observed slumps occur at depths below the influence of surface water waves, they do not consider the possibility that longer period waves, such as seiches or internal waves, may influence bed forms. It is instructive to investigate the theoretical seiche⁴ for any lake under sedimentological investigation to see if water velocities expected at the lakebed may be great enough to cause sediment movement. I have done this for a water body of rectangular cross-section (length 2,500 m, depth 30 m) as a crude model of the Stank Basin⁵ of Loch Lubnaig and conclude that a water particle velocity of 5 cm s⁻¹ is possible for a seiche of only 0.1-m amplitude, the period of oscillation being ~5 min. Seiche observations by Gill⁶ on Llyn Gwellyn, a lake of comparable size to the Stank Basin, suggest that a real seiche would be of longer period than the approximate theory predicts, but that particle velocities at the base of the water column would still be sufficient to transport silts and finer grains (Graf and Acaroglu⁷) even if the seiche amplitude were only a few centimetres. We² have observed three sediment waves or mounds in the bed of the Stank Basin. They rise, with gradients of 1:5, to a height of 5 m above the general level of the fine lakebed sediment, which is itself no more than 5 m thick. The sediment waves are symmetrical and show layering which parallels the sediment-water interface. Such layering and symmetry would not arise from slumping, but could be produced by a seiche-driven oscillatory water current. McManus and Duck¹ have drawn attention to the effect that sediment disturbance from slumping will have on the interpretation of lakebed sediment cores retrieved for palaeomagnetic or palaeoecological purposes. Disturbance in the manner I propose will be a more subtle effect because of the presence of seemingly undisturbed layers.

In Scottish lochs, the use of modern acoustic equipment (with the exception of

its largely ludicrous use in Loch Ness) has been neglected. This is not the tribute that Murray and Pullar⁷ would have wished. As well as conventional sidescan and profiling records, sediment acoustic velocity determinations are required^{8,9} to help assign proper depth scales and to assess sediment gas content, cited by Monroe¹⁰ as an important factor in causing slumping. I hope that McManus and Duck¹ and others will receive support to extend their work to other lochs and perhaps to map more completely the mounds in the Stank Basin and to verify if they are indeed associated with seiche activity induced by the winds howling down the glen at Ardochullarie Mor.

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DUCK AND MCMANUS REPLY—We welcome the comments of McKay concerning our recent contribution¹. He is correct to draw attention to sonar work in the North American Great Lakes. In view of their enormity these water bodies behave as inland seas and are not directly comparable with even the largest of the Scottish lochs.

Our contribution¹ specifically referred to sidescan sonar surveys and not to the use of sonar devices in general. The principal work reported was undertaken on Loch Earn and mention was made of Loch Lubnaig to illustrate that the subaqueous landforms recognized are not mere curiosities confined to one water body. We have since detected them in other lochs.

We were unaware of the boomer seismic work of McKay and McEwen² in Loch Lubnaig as their brief abstract was published in a Canadian journal not known for its contributions to Scottish environmental studies. The report of sediment waves or mounds in the Stank Basin² is interesting but, despite re-examination of sonographs and echograms³, we have been unable to confirm their presence due to lack of information regarding their position or orientation. However, marginal

spurs are recognized extending into the basin floor from the bounding slopes.

Seiches or internal waves may indeed influence loch bedforms. However, as yet, we have insufficient hydrodynamic data to corroborate this. Furthermore we have reservations about the seiche-associated current velocities calculated by McKay for Loch Lubnaig. Although we have not undertaken seiche observations in the loch there is good reason to believe that the 0.1-m surface seiche amplitude used in McKay's model, and to which the horizontal current velocity is proportional⁴, is excessively large. Chrystal⁵ demonstrated that seiche activity is poorly developed in Loch Lubnaig due to its shallow nature (mean depth, 13 m), its very irregular bottom and its orientation across the path of atmospheric disturbances. During a 6-week period only four instances of definite, but short-lived, seiche activity, with a period of about 24 min, were recognized⁵. For most of the period "... nothing was found but wind embroidery and sub-permanent wind denivelation, such as would be naturally expected in a shallow lake"⁵. Moreover, the maximum seiche amplitude observed by Chrystal⁵ (Fig. 22) was only about 0.5 cm. On the basis of McKay's model for the Stank Basin the maximum current velocity, at a seiche node, associated with such activity would be less than 0.3 cm s⁻¹. Average current velocities would be about half this value and tend to die away rapidly with time⁶. Thus currents resulting from surface seiches are unlikely to be responsible for the bedforms described.

To our knowledge the thermal behaviour of Loch Lubnaig has not been investigated. However, it is likely that internal wave (internal seiche⁷) activity will occur in association with summer stratification. Horizontal current velocity components generated by such water movements are known to be up to five times greater than those associated with corresponding surface seiches^{8,9}. Moreover, these currents can persist for several days⁸. Hence it is possible that internal waves may have a role in the formation of the structures recognized by McKay and McEwen², perhaps in the manner advocated by Mortimer¹⁰.

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Control of fertility in red deer

LOUDON *ET AL.*¹ have recently invoked a suckling-mediated mechanism for the control of fertility in red deer hinds. We suggest that their emphasis on the role of prolactin is not justified by their data and that other explanations are equally likely.

Although plasma prolactin concentrations are positively associated with the frequency of suckling², the authors fail to show a significantly higher plasma prolactin level after 80 days, between groups of hinds on low versus high planes of nutrition, despite the persistence of high suckling frequencies in the former. There is a minimum lag of some 30 days separating this latest detection of a difference in plasma prolactin levels between the groups and the earliest possible conception on day 110—when the stag was introduced. It is questionable as to whether there would be an ovulation-inhibiting effect of increased plasma prolactin levels this far in advance of ovulation, even if, as has been suggested³, such an effect were to operate on the follicular stage of the 18.3 day oestrous cycle⁴. This is assuming that prolactin has a causal influence on oestrous cycle activity which itself remains in doubt⁵.

Loudon *et al.* do not attempt to separate from the suckling data the confounding effect of differences in mean body weight between the two groups. It is well established that body weight not only influences fertility rates but may also affect the onset of oestrus in mammals⁶. In farmed red deer small hinds calve later than heavy hinds: a 1 kg increase advancing calving by between 0.3 and 1.0 days (ref. 7 and A.S.I. Loudon, personal communication). Furthermore, in the wild the average non-lactating hind calves approximately 2 days earlier for every 1 kg increase in mean autumn body weight⁸. Therefore a 4.1 kg difference in mean body weights between the two groups could explain the 6.5 day difference in return to oestrus and could be tested with their data. However, even if this is plausible and weight for weight hinds on a low nutritional plane return to oestrus later, this may be for reasons other than lactational control. For example, it is well known that in sheep, which cease lactation many weeks before the rut, body condition interacts with the plane of nutrition immediately before breeding⁹. At a

given weight individuals that are rapidly improving in condition, as might be expected in the group of hinds on permanent grass pasture, show earlier ovulation and higher ovulation rates.

In summary, Loudon *et al.* concur with at least one other study in failing to show any influence of the plane of nutrition on plasma prolactin levels during or immediately before the onset of the reproductive period¹⁰. As a consequence, they have little reason to invoke elevated levels of prolactin as a mechanism delaying conception in poorly nourished hinds. Finally, by not controlling in any way for maternal body weight and/or condition, the major confounding variable is not eliminated nor its contribution to the observed effect even brought into question.

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LOUDON *ET AL.* REPLY—In our paper we drew attention to the influence of pasture type on the milk yield, suckling patterns and fertility of red deer hinds. We were alerted to the physiological implications of the differences in suckling pattern when we considered our data on prolactin. These data, in common with a number of other studies, showed that prolactin levels were correlated with suckling frequency. At no point in our paper did we suggest that prolactin *per se* was ultimately involved in the regulation of fertility in red deer. Indeed we would suggest that in seasonally breeding mammals such as deer and sheep prolactin may have little influence on fertility. Thus, sheep treated with bromocriptine to block the normal high levels of prolactin in mid-summer resumed fertility at an identical time to untreated control sheep^{1,2}. Thus although prolactin may have an important function in regulating fertility in some mammals including humans, we agree with Albon and Iason that there is little evidence that

it may be directly important in the case of red deer.

We know of only one study that attempted to control female weight, food intake, milk yield and offspring growth rate while manipulating suckling frequency. In this study, ewes compelled to suckle their offspring twice a day resumed oestrous activity at an earlier date than those permitted to suckle five times a day³. We acknowledge that there is clear evidence that the body condition of non-lactating sheep prior to mating has an effect on their fertility but suggest that in the cases of wild mammals, including red deer, which, unlike sheep, frequently suckle their offspring through the mating period, the pattern of suckling activity may have a profound effect on their subsequent fertility. Body weight *per se*, like prolactin, simply correlates with differences in return to oestrus in red deer. We are unable to attribute differences in body weight between the two groups of hinds to differences in body condition or differences in gut fill associated with the complex effects of grazing different grass pastures.

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Matters Arising

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Super theories

Abdus Salam

Supersymmetry and Supergravity. By Julius Wess and Jonathan Bagger.
Princeton University Press: 1983. Pp.180. Hbk \$40, £34.50; pbk \$12.50, £10.90.

SUPERSYMMETRY — a possible symmetry between bosons (particles of spins zero or one or two, obeying Bose statistics) and fermions (particles of spins $\frac{1}{2}$ or $\frac{3}{2}$ obeying Fermi–Dirac statistics) — is an incredible postulate. It could have been made at any time after 1935, when the canons of quantum theory of fields were established. We had, however, been brought up from the kindergarten to think of spin zero (or spin one or spin two) bosons as intrinsically different from spin $\frac{1}{2}$ (or spin $\frac{3}{2}$) fermions. The fermions are individualists. In accordance with Pauli's exclusion principle, no two identical fermions could congregate at the same space point. In contrast the bosons are gregarious. They like to congregate and seek each other's company at the slightest provocation. How could one even try to place such different objects in the same multiplet of a possible symmetry group? In what sense could there be a symmetry between quanta of matter (typically fermions) and quanta of forces (typically bosons)?

In 1971, in the USSR, Golfand and Lichtman first discovered that one could, if one wished, introduce a fundamental symmetry between bosons and fermions — notwithstanding their intrinsic differences. The significance of their work was totally missed, however, and ignored until 1973 when this same symmetry was rediscovered by Julius Wess and Bruno Zumino working in the West. Even then (and despite the self-evident elegance of the symmetry, the relative freedom of supersymmetrized Lagrangians from field-theoretical infinities, as well as the guaranteed positivity of the energy) there was no direct evidence of the symmetry's existence at energies available in laboratories and supersymmetry remained a minority interest. The esoteric nature of the subject was reflected by the complete absence of relevant textbooks; conference proceedings and excellent review reports, yes, but no pedagogical material.

Now, after several years of increasing interest, a new generation of texts has begun to appear. Wess and Bagger's *Supersymmetry and Supergravity*, based on a course of lectures given by the senior author at Princeton, is one of the first. To assess the book, what it contains and what it chooses to leave out, it is necessary to consider the recent history of ideas in this field.

Two of the early developments concerned, first, the recognition of the existence of the so-called extended super-

symmetries ($N = 2, 3, 4 \dots 8$) where internal symmetries are married to simple supersymmetry ($N = 1$); and second, the superspace and the superfield method of exhibiting $N = 1$ supersymmetry in field theory. The simple $N = 1$ supersymmetry admits of two spin zero particles being grouped together with one spin $\frac{1}{2}$ Majorana particle into a single "supermultiplet". All these particles must possess the same mass. The $N = 1$ superfield is a field-function which describes this entire set of particles; the field is defined in "super-space" which is space-time manifold augmented with an additional fermionic coordinate. The extended $N = 2$ supersymmetry admits of a supermultiplet with four spin zero and two spin $\frac{1}{2}$ objects. The super-space this supermultiplet would exist on would admit of two fermionic coordinates. And so on for $N = 3, 4, \dots$, this sequence ending finally with the $N = 8$ supersymmetry — the so-called maximal supersymmetry — which would group together the following into its fundamental supermultiplet: one spin two, eight spin $\frac{3}{2}$, twenty-eight spin one, fifty-six spin $\frac{1}{2}$ and seventy spin zero massless objects.

During 1974–1975 renormalizable supersymmetric theories of spin $\frac{1}{2}$ and spin zero objects were elaborated, as were supersymmetric theories of spin one and spin $\frac{1}{2}$ particles, describing gauge quanta and their supersymmetric partners, the gauginos. Then, in 1976, the prize problem of supersymmetrizing gravity was solved. This was the prize problem because a gauging of supersymmetry must inevitably lead to a supersymmetrization of Einstein's gravity. The $N = 1$ "supergravity" which results is a generally-covariant, local, supersymmetric theory of the spin 2 particle (the hypothetical graviton) and of its supersymmetric partner (the still-more-hypothetical gravitino of spin $\frac{3}{2}$). The following years were an exuberant period of writing down of theories of extended supergravities ($N = 2, 3, 4, 8$). With these — and particularly with $N = 8$ supergravity — a singular hope was born: that particles of spin two and spin one, mediating *all* the four fundamental forces (gravity, electroweak and strong), together with the appropriate spin-zero Higgs needed to break the electroweak and other gauge symmetries, and interacting with "source" matter of spin $\frac{1}{2}$ quarks and leptons, could all be described by the fundamental supermultiplet of the $N = 8$ theory interacting with itself. This would not only unite the "marble" of gravity with the "base wood" of matter, as Einstein

dreamt; in Hawking's eloquent phrase, it would be the "Theory of Everything".

Unfortunately, the spectrum of particles, even in the maximally extended supergravity theory ($N = 8$) turned out to be inadequate to accommodate the known quarks, leptons and gauge particles. It was also soon recognized that, after gauging, any such theory must contain two coupling constants — the Newtonian constant determining the strength of the gravitational force and an unacceptably large cosmological constant in lieu of the fine structure constant (phenomenologically wrong by factor 10^{120}). On the technical side too, there were problems. It came to be clearly appreciated that the geometrization of physics, which started with the Einsteinian revolution of associating gravity with the curvature of space-time, would not be complete until supergravities had been described in terms of geometrical entities (super-curvature and super-torsion) in extended space-times — extended with the addition of the new fermionic coordinates on which the requisite superfields depend.

This was the situation in 1981. We had theories which were tantalizingly elegant but technically incomplete; theories which were relevant, but not quite adequate as far as the known low-energy physics was concerned. Since 1981, however, a fresh look has been taken at supersymmetry theories. The motivation for this has come from a new direction — from the so-called hierarchy problem, the problem of disparate mass scales in physics. In particle physics the relevant scale of masses ranges from the mass of the electron (of around 10^{-3} GeV) to the Planck mass associated with Newton's constant of gravity ($\approx 10^{19}$ GeV). The ratio of these two masses is an enormous number of the order of 10^{22} . And if the electronic neutrino is also massive (with an expected mass of the order of 10^{-8} GeV), the range is even more staggering. Dirac pointed out this problem of large numbers a long time ago; he attempted to solve it within the context of his cosmological theories, with a time-varying gravitational constant. Such theories, however, appear not to be favoured by current experiment.

Around 1981, came the realization that at least one aspect of this problem — the radiative stability of the mass ratios — could be resolved using supersymmetric ideas. With this input, suppose one was not too ambitious and did not ask for a Theory of Everything in one go; suppose one were to go back to 1974 and 1975 and try to invent a humble, realistic supersymmetric model of the electroweak or the electroweak theories only. One would choose to eschew gravity and with it the spin $\frac{3}{2}$ superpartner of the graviton. Such a model theory would contain photons and photinos; Ws and Zs, and their superpartners the winos and zinos; it would contain higgs and higgsinos. It would also contain quarks and squarks, and leptons

and leptinos (quarkinos and leptinos would be spinless; photinos, winos, zinos and higgsinos would carry spin $\frac{1}{2}$). How near to a successful model could one come in this restricted programme?

To be physically realistic, the most crucial further input needed would be a mechanism for supersymmetry breaking. This would generate a mass-difference between the known particles and their (hitherto undiscovered) superpartners, and would explain our inability in not having detected these superpartners so far. A desirable supersymmetry-breaking mechanism should be spontaneous, if only to conform to the pattern of symmetry breakings already familiar in particle physics.

But such a spontaneous supersymmetry breaking (even if it could be simply accomplished) would pose its own problems. It would lead to the existence of at least one massless spin $\frac{1}{2}$ particle — the goldstino. Could this massless fermion be one of the known neutrinos? Alas, the predicted low-energy behaviour of the goldstino turned out to be quite different from that of neutrinos. The goldstino must be suppressed.

Now the only known mechanism to suppress the appearance of the goldstino is to re-introduce the spin $3/2$ gravitino, which would absorb the goldstino (super-Higgs effect) and itself become massive. We are inevitably being led back to supergravity, but this time with supersymmetry-breaking as an important new ingredient. This is not a unification of gravity with other forces (electroweak or electro-nuclear). But it is a triumph nevertheless, in showing that gravity may already be influencing low-energy particle physics.

Models incorporating these ideas were constructed during 1982. The exciting thing is that this type of supersymmetry-breaking (i.e. through the introduction of $N = 1$ supergravity) can also induce a breaking of the electroweak gauge symmetry. The masses of the W s and the Z s are then a consequence of supergravity and super-Higgs effect! The spin $3/2$ superpartner of the graviton is expected to weigh around 300 GeV. The winos and zinos may be lighter than their supersymmetric partners, the recently discovered W s and Z s. If experiments at the CERN $p\bar{p}$ collider show that the W s and the Z s do indeed decay into winos and zinos, the age of supersymmetry will have come. Further evidence for this may be provided by proton-decay experiments, currently in progress. In the supersymmetric models the favourite decay is likely to be the proton decaying into a muon plus a kaon rather than the non-supersymmetric decay into a positron plus pion (searched for hitherto and apparently not found). The future CERN Large Electron-Positron accelerator (now under construction for Z^0 physics) may, in fact, justify itself much more as a supersymmetry accelerator. My own hunch is that this is what will happen.

But even if $N = 1$ broken supergravity theory, in interaction with $N = 1$ supermatter, is indicated by low energy (a few hundred GeV) experiments, this would still not be a Theory of Everything. We would need to bring back the ideas of extended supergravity and in particular of the $N = 8$ extended theory to achieve this. What then of progress in this area?

Here also there have been developments of great potential significance. Extended supergravities are flourishing with the discovery (in 1979) that the maximal $N = 8$ supergravity theory in four space-time dimensions can be recovered from the simple $N = 1$ supergravity in a space-time of eleven dimensions. This is a super-extension of an original idea of Kaluza and Klein, formulated some 60 years ago, in which the following miraculous happening was noted: an Einsteinian gravitational Lagrangian in five dimensions is equivalent to the normal Einsteinian Lagrangian in four dimensions plus Maxwell's electromagnetism, when the fifth dimension is "compactified" to a vanishing size. The new version of this — the super-miracle — occurs in eleven dimensions, where the simple ($N = 1$) supergravity gives rise exactly to the $N = 8$ extended supergravity theory in four dimensions, when the extra seven dimensions are suitably shrunken.

But what if the compactification size is finite, albeit smaller than the Planck size (i.e. $< 10^{-33}$ cm)? What effect is the topology of the seven-dimensional "internal" manifold likely to have on the resulting physics? Do such compactifications yield new (broken) supertheories, more in accord with experiment than the original massless $N = 8$ was? This is the search that is currently on in the context of a fundamental Theory of Everything. Some day, it is hoped, this method of attack will embrace and meet the phenomenological approach mentioned earlier.

And what of Wess and Bagger's new book? It is a beautiful exposition of the original ideas of Wess and Zumino in formulating $N = 1$ supersymmetry and supergravity theories, couched in the language of superfields introduced by Strathdee and the reviewer. The use of differential forms in super-space leads to a succinct exposition of ideas of super-torsion and super-curvature. Not discussed, however, are extended supersymmetries, or extended supergravities. There is also no comparative discussion of other approaches to $N = 1$ supergravity (except that of Wess and Zumino); and, of course, there are no phenomenological applications. But within its compass, the book is masterly. Coming as it does from one of the originators of the subject, all serious students of particle physics would do well to acquire a copy. □

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Working plants for the ecologist

Peter D. Moore

Physiological Plant Ecology II, III and IV. (Vols 12B, C and D of *Encyclopedia of Plant Physiology*, New Series.)

Edited by O.L. Lange *et al.*

Springer-Verlag: 1983. Vol. 12B, pp. 747, DM 288, \$111.82; Vol. 12C pp. 799, DM 298, \$115.70; Vol. 12D pp. 644, DM 290, \$112.60.

THE publication of Vol. 12D in the new *Encyclopedia of Plant Physiology* marks the completion of the section dealing with the essentially ecological aspects of plant physiology. Since the appearance of Vol. 12A, the first in the set (for review see *Nature* 301, 181; 1983) which dealt largely with the physical environment, there have been two intervening volumes published.

Volume 12B covers two main topics, water relations and carbon assimilation. In fact, most of the photosynthetic material is actually dealt with in 12A and what remains here is closely related to water balance problems, for example the article on the ecological significance of C_4 metabolism by C.B. Osmond *et al.* Here the competitive advantages are considered mainly in connection with drought, though some mention is made of aquatic plants. On the water relations side, subdivision of the subject consists of the various stages of water passage through the plant and also the water balance of individual cells. Of general interest to ecologists are chapters on drought tolerance, flood tolerance, frost-drought and seed germination.

Volume 12C deals mainly with inorganic aspects of plant nutrition and the ecological orientation of material is here very apparent. Limestone and pH effects have their own chapter (H. Kinzel), as do metals (H.W. Woolhouse). Microbial studies also receive some attention, with chapters on halotolerance, lichen symbioses, nitrogen fixation and mycorrhiza. That macabre source of interest the carnivorous plant is also given its own chapter (U. Lüttge) and a timely plea is made for a quantitative, systems study of these well-worked but little understood plants.

Interactions between plants and other organisms forms a second theme in this volume, including such topics as parasitism, the ecology of viruses, pollination (a chapter well supplied with chemical information difficult to find in standard texts) and herbivory, where the phenomenon of compensatory growth is given welcome prominence. It is left to E.I. Newman to cover interactions between plants in just one chapter and he tackles this wide subject by concentrating on mechanisms of interference.

The final volume, 12D, is perhaps the

most remarkable in that it extends the coverage of the series far beyond the scope of earlier encyclopaedias of plant physiology. Its subject is the plant component of ecosystems. Beginning with chapters on nutrient cycling in terrestrial, freshwater and marine ecosystems, it focuses on productivity within the major biomes. The aquatic nutrient cycling chapters concentrate upon specific elements, such as phosphorus and sulphur, and the marine account ties in well with the plant-interactions theme of the previous volume by analysing the influence of nutrient levels on phytoplankton diversity.

The productivity chapters feature the technical problems associated with estimates of production and also provide some detailed accounts of the growth models used to describe production processes on a biome scale. The volume closes with chapters reviewing pollution and eutrophication problems, including

topical items such as increasing atmospheric CO₂ levels.

Looking back through the physiological plant ecology section of the *Encyclopedia*, one must be impressed by the amount of information which has been pressed into four volumes. The provision of a subject index at the end of the quartet will enable ecologists of all shades to gain rapid access to a wealth of up-to-date, well-reviewed material with extensive bibliographies. Subject overlap and repetition does exist, but is not excessive; omissions are few, though some topics, such as plant competition, deserved more space. Although somewhat bulky, these four books will render redundant about two shelves of older ecology texts, so it is well worth making room for them. □

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Fisher from inside

Cedric A. B. Smith

Natural Selection, Heredity and Eugenics.

Edited and introduced by J.H. Bennett.
*Oxford University Press: 1983. Pp.306.
£17.50, \$47.50.*

In a letter to Hooke, Newton wrote "If I have seen a little further it is by standing on the shoulders of Giants". But the pace of scientific advance has accelerated so that, as Gerald Holton is said to have remarked, "We are now uniquely privileged to sit side by side with the giants on whose shoulders we stand".

As a student, I knew a little about mathematical genetics, and was vaguely aware that the major pioneering work had been done by R.A. Fisher, J.B.S. Haldane and Lancelot Hogben. (I was only later to hear of Sewall Wright.) I had a rosy vision of Fisher, Haldane and Hogben frequently discussing, with great excitement, their latest discoveries and plans for the future.

In fact, after the Second World War, fate decreed that I should become a mathematical geneticist, and get to know Fisher, Haldane and Hogben personally (and even, on occasion, sit side by side with them). What immediately became clear was that relations between them were alas far from ideally cordial and that each in his own way could sometimes be inexplicably prickly, hindering both close intellectual contact and scientific advance.

The publication of this book, which mainly consists of correspondence to and from Fisher, does much to throw light on this sensitivity, on the relationship between the three men and especially on the character of Fisher himself. With his closest friends, Fisher was always helpful and generous — perhaps his touchiness

with others was a barrier put up in self-defence, or a reaction to the severe disappointment when many of his outstanding papers produced when he was young had been turned down by major scientific journals. In his letters we see the real Fisher, almost always gentle, polite, friendly and idealistic, and most willing to listen to argument. Rather than the almost impenetrable and tortuous prose of much of Fisher's scientific writings we have instead a limpid expression of his thoughts.

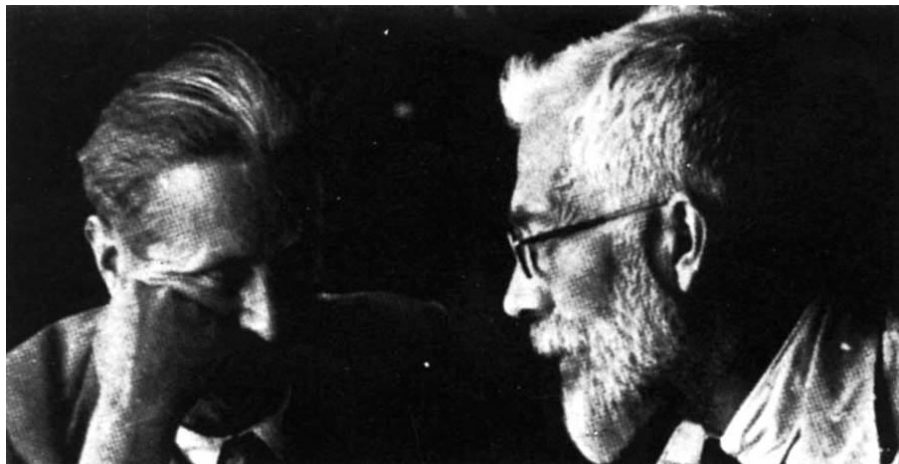
Two other points emerge from the correspondence printed here. The idea of evolution through selection acting on rare mutation, which from around 1935 seemed obvious common sense, and the consequent mathematical theory, had achieved this position of seeming to be the most logical and proper approach to evolution only quite recently by reason of the work of Fisher, Haldane and Hogben. And the unnecessarily strained relations between the three protagonists were largely of recent origin — a few years earlier friendship and helpfulness had prevailed.

There are occasions when a new branch of knowledge seems to be born suddenly,

rather than by a process of gradual accretion. Fisher's 1918 paper "On the Correlation between Relatives on the Supposition of Mendelian Inheritance" must have been one, and the appearance in 1921 of his book *Statistical Methods for Research Workers* another. The paper on correlations between relatives covered the topic so thoroughly that not a great deal has been added to the conclusions in the 64 years which have followed. It appears that we owe the appearance of this paper to the constant encouragement of Major Leonard Darwin (Charles Darwin's son) who corresponded regularly and frequently with Fisher for 27 years. In their letters important insights emerged, some of which did not find their way into Fisher's published papers. For example, William Hamilton has attributed the inspiration of his theory of the evolution of altruism to remarks by Haldane. But in a letter to Darwin on 27 June 1929, Fisher shows that he had understood the essential point already, namely that an organism's willingness to sacrifice itself may nevertheless improve the chances of survival of the genes it carries because they will also be found in close relatives.

Just over a hundred pages of the book are occupied by the Darwin-Fisher correspondence, and approximately another hundred are taken up by extracts from letters on selection and evolution, including letters to (and sometimes from), W.C. Bond on blood groups, L.C. Dunn on dominance, E.B. Ford on selection in butterflies, J.B.S. Haldane on miscellaneous topics, J.S. Huxley on Family Allowances and S. Wright on topics in the mathematics of selection. The book also contains some other papers by Fisher which are unpublished or not readily accessible. In all, it illuminates in an interesting and informative way the development of the theory of natural selection during the years 1910-1960, both as regards Fisher's own personal contributions and his reactions to those of other pioneers. □

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R.A. Fisher (right) with C.D. Darlington at a genetics conference in Stockholm, 1948.

End of blue-green algae

Sergey V. Shestakov

The Biology of Cyanobacteria.

Edited by N.G. Carr and B.A. Whitton.

Blackwell Scientific/University of California Press: 1982.

Pp.688. £42, \$75.

It is now unarguable that phototrophic organisms, which have for over a century been called blue-green algae, belong to the prokaryotes and are thus the property of microbiologists rather than botanists. The best proof of this is the appearance of an authoritative book with the word "cyanobacteria" in its title. The term has thus been fully legalized. In the foreword, written shortly before his death, Roger Stanier succinctly defined them as: "Micro-organisms that harbour within a typically prokaryotic cell, a photosynthetic apparatus closely similar in structure and function to that located within chloroplasts of phototrophic eukaryotes".

The book is a successor to *The Biology of Blue-Green Algae*, published by Blackwell Scientific and University of California Press in 1973, and clearly reflects the progress in cyanobacteriology made in the intervening decade. The contributors are all leading experts and between them provide comprehensive reviews of the morphology, physiology, biochemistry and ecology of the cyanobacteria, and (in the final chapter) of palaeomicrobiological aspects of their origin and evolution.

The role of cyanobacteria in freshwater and marine ecosystems is described in great detail, and the peculiarities of structure and function of membranes and cell wall, heterocysts and akinetes, phycobilisomes and gas vesicles are discussed in a new light. A lot of attention is also paid to photosynthesis, carbon, nitrogen and phosphate metabolism, and to motility and taxis. Particularly stimulating are the chapters on genetics and molecular evolution, in which the genome of cyanobacteria and their phylogenetic position within the

prokaryotes and in relation to eukaryotes are for the first time exhaustively analysed. The genetic relatedness of cyanobacteria and plastids of eukaryotic photosynthesizers is well argued.

The book is not without its disappointments, however. Cyanoviruses, symbiotic systems and soil ecology of cyanobacteria are not included, while the omission of certain contemporary issues in applied biology — the use of cyanobacteria in agriculture and biotechnology, for example — is especially regrettable. (Cyanobacteria are promising subjects for the genetic engineer, and may in time provide the clues by which we unravel the intricacies of such fundamental processes as nitrogen fixation, membrane biogenesis and cell differentiation.) The editors have in general done an excellent job; but some of the authors, although giving an impressive

show of their knowledge, have sadly failed to give pointers to the immediate research problems to be tackled in their specific areas.

But the plus points easily outweigh the minus. The book is extensively illustrated and thoroughly referenced, even including very recent data, which will make it a good source for advanced students and new research workers. And since cyanobacteria are now attracting an increasing number of scientists from a variety of backgrounds, this highly sophisticated book will certainly be appreciated not only by microbiologists, but also by biochemists, cytologists, ecologists, molecular biologists and other specialists.

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Algebraic astronomy

P.K. Seidelmann

Vectorial Astrometry.

By C.A. Murray.

Adam Hilger, Bristol/Heyden,

Philadelphia: 1983. Pp.353. £25, \$49.

THE lack of a comprehensive yet readable text on spherical, or practical, astronomy is generally recognized. Instead, a number of good, specific-purpose texts*, and several, much older textbooks† which are still valuable reference works when wisely used, have had to suffice.

While astrophysics has changed drastically in the last century, astrometry has changed by degree. Thus, an astrophysics textbook from the last century would be wrong, but a spherical astronomy volume from the nineteenth century would only omit recent developments and lack the accuracy of current standards. This evolutionary progress in astrometry may explain the previous absence of a textbook based on vector and matrix algebra.

C.A. Murray's *Vectorial Astrometry* is not the much-needed comprehensive, readable text. Rather it attempts to apply vector and matrix algebra systematically to spherical astronomy. At the same time the International Astronomical Union reference system, time scales and constants, introduced at the beginning of 1984, are explained and used throughout the volume.

The personality and interests of Andrew Murray are accurately reflected by the

strengths of the book. Photographic astrometry, relativistic astrometry, the theory of refraction, precession and nutation are well presented. For many subjects the latest developments are included and the vectorial and matrix equations are derived.

The volume would be significantly improved by good diagrams, however. The present two-dimensional, captionless line drawings add little to the clarity of the treatment. On subjects that are not associated with previous interests of the author, there is little in the way of descriptive explanation; the purpose appears to be the derivation of the necessary equations rather than communication of their reason and purpose. Several chapters contain individual sections without any apparent connection between the subjects. Also, I suggest reading the appendices first, so the notation and basis of some equations are understood.

While many new textbooks provide the cookbook formulae and methods for performing specific computations, this volume emphasizes the theory and derivation of equations and avoids the practical applications. Thus, under the title of astrometry through the atmosphere, the theory of refraction is discussed in great detail, but there is no mention of practical methods of calculating refraction.

For those who believe that astrometry has never entered the twentieth century, this is the book to change their minds. If you wish to apply vector or matrix algebra to spherical astronomy, if you need the relativistic formulation of astrometric calculations, and if you are interested in the latest developments and most accurate methods of astrometry, you will find this book to be interesting and useful. For all astrometrists, and for astronomers who must make accurate spherical astronomical computations, it will be an important reference volume.

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In colour

Colour Vision: Physiology and Psychophysics, a book stemming from the conference on the subject held in Cambridge, UK, in 1982, has recently been published by Academic Press. Editors are J.D. Mollon and L.T. Sharpe; price is £28, \$49. Issues raised at the conference were discussed in a News and Views article in *Nature* 300, 320; 1982.

Life dictionary

A new edition of E.A. Martin's *Dictionary of Life Sciences*, which first appeared in 1976, has been published by Macmillan Reference Books, London. According to the editor, revision of the *Dictionary* has taken account of advances in genetics, molecular biology, microbiology and immunology, while coverage of the more traditional aspects of biology has been reduced slightly. Price is £22.50.

*e.g. I.I. Mueller's *Spherical and Practical Astronomy as Applied to Geodesy* (Frederick Ungar, 1969); E.W. Woolard and G.M. Clemence's *Spherical Astronomy* (Academic, 1966); L.G. Taff's *Computational Spherical Astronomy* (Wiley, 1981); P. Duffett-Smith's *Practical Astronomy with your Calculator* (Cambridge University Press, 1979).

†e.g. S. Newcomb's *A Compendium of Spherical Astronomy* (Macmillan, 1906/Dover, 1960); W. Chauvenet's *A Manual of Spherical and Practical Astronomy* (Lippincott, 1863/Dover, 1960); W.M. Smart's *Textbook on Spherical Astronomy* (Cambridge University Press, 1931).

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